بسم الله الرحمن الرحيم

Effects of Intranasal Immunization and Mucosal Adjuvants on the Immunogenicity of *Pasteurella multocida* in Rats.

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صدق الله العظيم

سورة يوسف(الآية ٧٦)

Dedication

This work is dedicated

To

My parents, Brothers, sisters and Friends

With deep love

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On the accomplishment of the present study, I would like to take this opportunity to extend my deepest sense of gratitude and words of appreciation towards those, who helped me during the pursuit of study. I deem it a proud privilege and feel immense pleasure to acknowledge all those who are directly or indirectly involved.

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Abstract

This study was carried out to study the effects of intranasal immunization, mucosal adjuvants and vaccine-adjuvant ratio on the immunogenicity of *Pasteurell multocida* in rats. Forty-eight rats were kept in a conventional animal house and allowed to adapt to the environment, food and water for one week. They were assigned to one of five groups (G1 to G5). G1, G2 and G3 were further divided into subgroups (a) and (b) each composing 6 rats. Groups 4 and 5 were control groups of 6 rats each. The rats were immunized intranasally with *P. multocida* broth bactrin mixed with mucosal adjuvants at day one and day 15. Mucosal adjuvants used were olive oil for group 1, sesame oil for group 2 and vitamin D3 for group 3. The vaccine adjuvant ratio for all a subgroups was 1:1 and 2:1 for all subgroups b. Rats in group 4 received plain *P. multocida* broth bacterin and rats in group 5 were sham-vaccined with phosphate-buffered saline (PBS). All rats were sacrificed on day 21 post initial immunization. Lung and trachea from each rat were carefully removed and the trachea and bronchial tree were washed thoroughly with 5 ml PBS. The tracheobronchial (TB) lavage was collected, centrifuged and the supernatant was kept at -20 ºC for the detection of *P. multocida* specific mucosal Ab using gel diffusion precipitation test (GDPT) and indirect haemagglutination (IHA) assay. *P. multocida* whole cell lysate (WCL) was used as antigen for GDPT and for coating formalized tanned RBCs. In GDPT, precipitation lines were obtained using TB lavage from all *Pasteurella multocida* intranasally immunized rats, and relatively sharp precipitation lines were obtained in TB lavage of rats of G1b, G2a and G3a, indicating the presence of *P. multocida* specific Ab in their TB lavage. The IHA was performed using two-fold dilution of TB lavage in v-shaped microtitre plates, starting with 1/200 dilution. Significant

(P<0.05) titre were obtained in TB lavage of G1b, G2a and G3a. The IHA titre was expressed as the reciprocal of the highest dilution of TB lavage showing a diffuse matt of erythrocytes as compared to negative control showing a clearly delineated spot of erythrocyte within the vertex of the well. Rats of G1b that received *P. multocida* broth bactrin in olive oil in a ratio of 2:1 gave high Ab activity in TB lavage as was determined by IHA assay compared to ratio of group 4 (Adjuvant control) that received plain broth bactrin vaccine. This difference is highly significant (P=0.0082). Rats in this group G1b showed also higher Ab activity in their TB lavage than rats in group 2b that received *P. multocida* broth bactrin in sesame oil in a ratio of 2:1 (P=0.9991) as well as to rats in G3b (P=0.9661). On the other hand, rats in group 2a that received *P. multocida* broth bactrin in sesame oil in a 1:1 ratio showed high Ab activity in their TB lavage as determined by IHA assay. The Ab titre of this group 2a was significantly higher (P=0.0227) than in rats of G2b, G1a and G3b. Similarly, rats in G3a that received *P. multocida* broth bactrin in vitamin D3 in the ratio of 1:1 had significant Ab titres in their TB lavage, in comparison to G4 with P values equal to 0.0272. In addition, sesame oil and vitamin D3 in 1:1 ratio with *P. multocida* broth bactrin had induced high Ab activity at the respiratory tract, whereas olive oil vaccine-adjuvant ratio of 2:1 induced Ab activity of the same magnitude. It seems that the booster dose was necessary to ensure a prolonged and strong local immune response for *P. multocida* broth bactrin. Since the effectiveness of this broth bactrin vaccine was expected to be much lower than live vaccine, mucosal adjuvants as potent delivery systems for antigens were incorporated to enhance the uptake of the vaccine-antigen and ameliorate the induction of local Ab response at the respiratory tract as measured by IHA and GDPT assay.

المستخلص

أجري هذاالبحث لدراسة مستضدية بكتريا الباستيوريلا متعددة القتل عن طريق التحصين بالأنف وتأثيرالمساعدات المناعية المخاطية ومعدل اللقاح للمساعدات المناعية في الجرذان الممنعة ضد بكتيريا الباستيوريلا متعددة القتل. حفظت ثمانية وأربعون جـرذا لمـدة أسـبوع بحظيـرة الحيوانات التقليدية وذلك للتكيف على البيئة و الغذاء و المـاء. قـسمت الجـرذان إلـي خمـس مجموعات (ج1-ج5).المجموعة الأولى و الثانية و الثالثة قسمت إلي تحت مجموعات أ و ب، كل منها بها 6 جرذان. المجموعة الرابعة و الخامسة كمجموعتي شاهد كل منهـا بهـا 6 جـرذان. حصنت الجرذان عن طريق الأنف بلقاح الباستيوريلا متعددة القتل الـذي تـم مزجـه بواسـطة المساعدات المناعية المخاطية في اليوم الأول و اليوم الخامس عـشر. اسـتخدمت المـساعدات المناعية المخاطية الاتية: زيت الزيتون للمجموعة الأولى و زيـت السمـسم للمجموعـة الثانيـة وفايتمين هـ3 للمجموعة الثالثة. معدل اللقاح بالنسبة للمساعدات المناعية المخاطية كان كـالآتي 1:1 لتحت مجموعة أ و1:2 لتحت مجموعة ب. حقنت مجموعة الجرذان الرابعة بلقاح بكتيريـا الباستيوريلا متعددة القتل و مجموعة الجرذان الخامسة تم حقنها بمحلول ملح الفوسفات المتعادل. شرحت كل الجرذان في اليوم الحادي والعشرين من بداية التحصين . أزيلـت الرئـة والقـصبة الهوائية بحرص ثم غسلت بواسطة 5 مل من محلول ملح الفوسفات المتعـادل. جمعـت نـواتج الغسول الرغامي الشعبي واخذ طافي الغسول بعد الطرد المركزي و حفظ فـي درجـة حـرارة 20 -° م وذلك للكشف عن الأجسام المضادة المخاطية النوعية لبكتيريا الباستيوريلا متعددة القتل واختبـار الـتلازن الـدموي الغيـر (GDPT)باستخدام اختبار الانتشار الترسيبي الهلامـي

. استخدمت خلايا بكتيريا الباستيوريلا متعددة القتل المحللـة كمستـضد لاختبـار (IHA)مباشر الانتشار الترسيبي في الهلامة ولتغليف كرات الدم الحمراء المعالجة بحمض التانك والفورمـالين. أظهر اختبار الانتشار الهلامي خطوط ترسيب وذلك عند استخدام نواتج الغسول الرغامي الشعبي في كل الجرذان الممنعة ببكتيريا الباستيوريلا متعددة القتل بواسطة الأنف. و حصل على خطـوط ترسيب بصورة واضحة نسبيا من نواتج الغسول الرغامي الشعبي لجرذان تحت مجموعة ج1ب و ج2أ و ج3أ وذلك لوجود الجسم المضاد النوعي لبكتيريا الباستيوريلا متعددة القتـل فـي نـواتج الغسول. اجري اختبار تلازن الدم الغير مباشر باستخدام التخفيف المـضاعف لنـواتج الغـسول ابتداء بالتخفيف 200/1. تم الحصول v الرغامي الشعبي في أطباق التلازن الدموي ذات الشكل على معيار عالي المعنوية في نواتج الغسول الرغامي الشعبي في تحت مجموعـات ج1ب و ج2أ وج3أ. ويعتبر معيار اختبار التلازن الدموي الغير مباشر مقلوب لأعلى تخفيف من نواتج الغسول الرغامي الشعبي التي تظهر كوسادة منتشرة من كرات الدم الحمراء مقارنة بمجموعـة الـشاهد السالبة التي تظهر كبقعة متجمعة في قاع الطبق. الجرذان التي حقنت بلقاح الباستيوريلا متعـددة القتل مع زيت الزيتون بمعدل1:2 أعطت أعلى فعالية للأجسام المـضادة فـي نـواتج الغـسول الرغامي الشعبي مقارنة بمعدل المجموعة الرابعة التي أعطيت لقاح الباستيوريلا متعـددة القتـل أظهرت جرذان هذه المجموعـة معـدل أجـسام .)(P=0.0082فقط. وكان هنالك فرق معنوى في نواتج الغسول الرغامي الشعبي أعلى من جرذان تحت مجموعة (P=0.9991)مضادة معنوى ج2ب التي أعطيت لقاح الباستيوريلا متعددة القتل مع زيت السمسم بمعدل 1:2، بالإضـافة إلـى كما أن الجرذان في تحت مجموعة 2أ التي (P=0.9661).الجرذان في تحت المجموعة الثالثة ب

أعطيت الباستيوريلا متعددة القتل مع زيت السمسم بمعدل1:1أظهرت معدلات أجسام مناعية عالية في نواتج الغسول الرغامي الشعبي التي قيست بواسطة اختبار التلازن الـدموي الغيـر مباشـر، من الجرذان فـي (P=0.00227)ومعيار الأجسام المضادة لهذه المجموعة 2أ كان معنويا أعلى تحت مجموعات ج1أ وج2ب وج3ب. أما الجرذان في تحت المجموعة الثالثة أ التي أعطيت لقاح الباستيوريلا متعددة القتل مع فايتمين هـ3 بمعـدل 1:1 فقـد أظهـرت أعلـى معيـار معنـوى في نواتج الغسول الرغامي الشعبي مقارنة بجرذان المجموعة الرابعة بالإضـافة (P=0.0272) إلى أن زيت السمسم وفايتمين هـ3 بمعدل 1:1المضاف للقاح الباستيوريلا متعددة القتل قـد حفـز إنتاج أجسام مضادة في القناة التنفسية، بينما زيت الزيتون المضاف كمساعد مناعي للقاح بمعـدل 1:2 قد حفز إنتاج أجسام مناعية بنفس الحجم. يعتقد أن الجرعة المنشطة كانت ضرورية لتأكيـد استجابة مناعية محلية قوية ومستمرة. ونظرا لان فعالية هذا اللقاح يتوقع أن تكون اقل من اللقـاح الحي فان المساعدات المناعية المخاطية، قد استعملت كأنظمة نقل فعالة للمستضدات، لتزيد مـن اصطياد مستضد اللقاح وتزيد من تحفيز الاستجابة المناعية الموضعية في القناة التنفسية المقاسـة و اختبار الانتـشار الترسـيبي الهلامـي(IHA) بواسطة اختباري التلازن الدموي الغير مباشر .(GDPT)

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INTRODUCTION

Haemorrhagic septicaemia (HS) remains a disease of major economic importance in the Sudan. In the Sudan, the disease is caused by *P.multocida* types B6 and E6 (Shigidi and Mustafa, 1979). Although the disease has been described since the turn of the last century, little is known about its pathogenesis. Virulence markers identified in other bacteria have not been well documented in *P.multocida*, apart from the fact that the capsule is essential for pathogenicity (De Alwis, 1990).

Although the disease was described originally as septicaemia, it is believed that the septicaemia in most instances is a terminal event and most likely it is preceded by a descending colonization of the respiratory tract by *P.multocida* causing an acute inflammatory response that might damage the alveolar septa permitting the bacteria to reach the blood circulation in high numbers fulminating in septicaemia. The instrumental inflammatory cells are activated alveolar macrophages. Death that follows is probably due to septic shock as in the case of other Gram negative bacteria. This has led to the hypothesis of pulmonary compartmentalization where a barrier is postulated that must be overcome before infection is systemically disseminated. Therefore, due to the sudden onset, brief duration and fatal nature of the disease, treatment in most cases is not possible and the only satisfactory and practical method of control and prevention is by carrying out timely vaccination of all healthy and in contact animals and adopting hygienic measures. Bacterins made from *P.multocida* have been available for many decades but with little effect in preventing the disease. Other major problems associated with these vaccines include: Generate systemic immune response rather than effective local immunity, occasional outbreaks in areas

covered by vaccination, produced post-vaccination shock, induce immune-enhancement following exposure to natural or experimental infection (De Alwis, 1990). Nonetheless, investigations into the immunologic potential of *P.multocida* over the last several years have led to the development of a variety of immunoprophylactic agents, but none of them has yet replaced the old bacterin vaccine (De Alwis, 1990). For most mucosal diseases of the respiratory tract, the induction of local immunity at the site of infection is crucial for optimal protection (Cox, Verdonck, Vanrompay and Goddeeris, 2006). In the immune animal, it is believed that the locally generated Abs of SIgA might abort early sequences of this disease to occur and hence SIgA confer protection against this disease without local or systemic involvement (Gerdts, Mutwiri, Tikoo and Babluk, 2006).

Since the effectiveness of HS bactrins vaccines is expected to be much lower than live vaccine, a potent adjuvant and delivery systems are needed to enhance the uptake of vaccine antigen and to increase the induction of immunity (Gerdts *et al.,* 2006). As a result, in this study intranasal route might be more favorable for inducing immunity at the respiratory tract, which constitutes the desired effector site for protection against haemorrhagic septicaemia.

The primary reasons for using intranasal route of immunization is that *P. multocida* infection affect or initiate the infectious process at the upper respiratory mucosal surface, hence mucosal application of the vaccine, preferably in mucosal adjuvant, is required to induce a protective immune response (Holmgren and Czerkinsky, 2005). Therefore, induction of local immunity at site of infection is crucial for optimal protection. The induction of SIgA represents the main effector mechanism of the local adaptive immune response, thus represents the primary goal for most mucosal

vaccines. Moreover, mucosal immunization induces both mucosal and systemic immunity with mucosal memory. If immunity wanes upon second exposure to *P. multocida*, there will be a rapid response both locally and systemically, (Cox *et al*., 2006). The principle objectives of this study were

1. To evaluate immunogenicity of *P. multocida* in rats.
2. To induce a local immune response at the respiratory tract using intranasal route.
3. To evaluate the effect of mucosal adjuvants in enhancing a local immune response.
4. To evaluate mucosal immunization in the generation of a local immune response.

CHAPTER ONE
LITERATURE REVIEW

2.1. Haemorrhagic Septicaemia

Haemorrhagic Septicaemia (HS) is a peracute, contagious and highly fatal septicaemic disease of both wild and domestic animals, with endemic nature in most parts of tropical and subtropical Asia and Africa (Bain, 1957).

2.1.1. Etiology

Hemorrhagic septicemia is caused by two serotypes of *P. multocida*; B 2 and E 2. The letter denotes the capsular antigen as determined originally by the indirect hemagglutination test of Carter (Carter, 1984), and the numeral 2 stands for the somatic or O antigen as determined by the agar gel diffusion precipitin test developed by Heddelston and associates (Namioka and Bruner, 1963). This somatic antigen 2 is the equivalent to the 6 in the classification of Namioka and associates. In a new classification, *Pasteurella multocida* strains causing most pasteurella infections, including hemorrhagic septicemia, are called *P. multocida* subspecies *multocida*.

2.1.2. Taxonomy of *Pasteurella multocida*

Pasteurella multocida (P.multocida) belong to the family *Pasteurellaceae* which contains the genera *Pasteurella, Actinobacilluss, Gallibacteria, Haemophilus, Lonepinella, Mannheimia* and *Phocoenobacter*.These genera share a number of common features and some organisms have been reclassified within these genera following deoxyribonucleic acid hybridization studies and 16 rRNA sequencing (Quinn, Markey, Carter, Donnelly and Leonard, 2002).

2.1.3. Cultural, Morphological and Biochemical Characterization

Pasteurella multocida species can be identified on the bases of cultural, morphological and biochemical characteristics. Topley and Wilson (1929) described *P. multocida,* as small, ovoid, Gram negative bacilli with bipolar staining characteristics, which are aerobic, facultatively anaerobic and produce indole and ferment carbohydrates with slight gas production.

2.1.4. Biotypes of *Pasteurella multocida*

Five biovars of *P.multocida* were proposed by (Carter, 1967) designated as biovar a (the mucoid biovar), biovar b (the haemorrhagic septicaemia biovar), biovar c (the porcine biovar), biovar d (the feline biovar) based upon hyaluronidase decapsulation, acriflavine folliculation, colonial iridescence, fermentation pattern, mouse pathogenicity, host predilection and serologic and immunologic characteristic. The fifth biovar is designated e (deer biovar) on the bases of its acid reactions in trehalose, sorbitol, mannitol and their failure to produce hydrogen sulphide (Frederiksen, 1973).

2.1.5. Antigenic structure and serotypes of *Pasteurella multocida*

Different methods, employing a wide range of antigenic components and cellular fractions were adopted to serotype *P.multocida* Roberts, (1947) classified strains of *P. multocida* into 4 serological types I, 11, 111 and IV by the passive mouse protection tests in which mice were passively immunized by antisera prepared against one strain and counter challenged by the homologous or heterologous virulent cultures of the strain. The colonial morphology and antigenic behavior of variants of *P.multocida* employing the S, R and M designation of Carter, (1957), using the acriflavine test revealed a characteristic reaction when freshly isolated type D *P. multocida* were subjected to the test. The test rapidly identified the non-immunogenic cultures of *P. multocida* together with the untypable ones. In

1955, Carter described a haemagglutination test for identifying *P. multocida* utilizing specific capsular antigens and capsular hyaluronic acid extracted by heating the cells at 56°C for 30 minutes in normal saline. These were adsorbed to human group (O) erythrocytes and positive results were detected by visible agglutination. The test recognized four different serological groups designated A, B, C and D and failed to allocate non encapsulated cultures to any specific group. When compared to Roberts type I, 11, III & IV they were found to be identical to type B, A, C and D, respectively. Carter, (1967) identified a strain of *P. multocida* isolated from cattle with an acute septicaemic disease which was different from that caused by serotype B *P. multocida* in cattle and buffaloes and designated it as serotype E . His serotype C gave inconclusive results in further studies and was dropped as a distinct serological group. (Rimer and Rhoades, 1986) identified an additional capsular serogroup designated serogroup F among *P.multocida* isolated from turkeys in USA with somatic serogroups I, 3, 7 and 12. Namioka and Murata (1961) studied the antigenic relationships between the somatic antigens (O-Ag) of *P.multocida* prepared by treating cultures of the organism with normal hydrochloric acid (N-HCL) and divided them into common and specific antigens. Cultures of type A and D were divided into two groups according to their O-Ag. Sub culturing of the organisms did not change the O-Ag into the rough form even if it was repeated for a year or more. They distinguished 6 somatic groups (1-6) among 24 strains of *P. multocida* from different parts of the world by means of absorption tests and consequently 6 serotypes were specified by combining their capsular types and somatic groups.

2.1.6. Epidemiology

The scope of epidemiology in modern animal husbandry practice is continuously widening. Epidemiological data provide information on various diseases which are pre-requisite for planning, execution and monitoring of disease control programmes. It is an important requirement for assessing economic impact of a disease and for developing disease forecasting system.

2.1.7. Hosts

De Alwis (1990) reported that the major animal species susceptible to HS were buffaloes and cattle. Buffaloes were found more susceptible than cattle and that in both species, young and adult animals were found more susceptible than older animals. Analysis of 26 recorded outbreaks of HS in buffaloes and cattle in various districts of Haryana (India) from 1995 to 1999 revealed higher disease prevalence in buffaloes than in cattle (Jindal, Kumar, Narang, Chaturvedi, Tomar and Garg, 2002).

2.1.8. Geographical Distribution of HS

An outbreak of HS is believed to begin when a 'latent carrier' animal becomes active and sheds virulent organisms infecting in-contact susceptible animals. Once a clinical case is established, dissemination of infection will increase and the magnitude of the outbreak that results will depend on the proportion of immune to non-immune animals in the herd (De Alwis, 1990). Hemorrhagic septicemia has a worldwide distribution and is endemic in Africa and Asia. In Africa, it was reported in most countries (Annosa and Isoun, 1975). The disease was found across Asia from the Middle East region to south eastern Asia (Anonymous, 1979).

2.1.9. Haemorrhagic Septicaemia in Sudan

The disease is endemic in Sudan and was reported nearly from all parts of the country. The disease was reported in Blue Nile province (1933), kassala (1939), Northern Kordofan (1943), and Upper Nile (1947). (Sud. Vet. Serv. 1933, 1939, 1943 and 1947). Serotypes B 6 and E 6 were isolated and identified from natural cases of Hemorrhagic septicemia in cattle by Shigidi and Mustafa in (1979). The disease was also reported as an acute pasteurellosis in camels in the Blue Nile province by (Hassan and Mustafa, 1985) and serotype B 6 was identified as the causative agent of the disease.

2.2. Pathogenicity and immunogenicity

Pasteurella multocida, a Gram-negative facultative bacterium, is one of the notorious animal pathogens causing widespread infections in various domestic animals; snuffles in rabbits, pneumonia and haemorrhagic septicaemia in cattles, sheep and goats and fowl cholera in chickens. In pigs, it causes severe respiratory diseases such as atrophic rhinitis (AR) and pneumonic pasteurellosis (Vasfi, Marandi and Mittal, 1997). Significant global economic loss in swine production due to growth retardation is caused by AR, and therefore, vaccination has merged as the most attractive and effective approach in controlling AR (Liao, Huangb, Hsuan, Chenb, Lee, Liu, Winton and Chien, 2006). Currently used vaccines, including inactivated and live vaccines, have their intrinsic problems as well as several advantages (Lubke, Hartmann, Schroder and Hellmann, 1994). A major advantage of live attenuated vaccines is that they are able to stimulate cross protection against different *P. multocida* serotypes. However, because the basis for attenuation is not known, it is not surprising that vaccinated flocks with live attenuated vaccines suffer outbreaks. Moreover, when a killed bacterium was used for vaccination, immunity is generally serotype-specific (Carpenter, Snipes, Kasten, Hird and Hirsh, 1991). The outer membrane of

Gram-negative bacteria contains lipopolysaccharides, several minor proteins and a limited number of major proteins present in very high copy numbers. One of the major outer membrane proteins, bacterial porins, functions as channels for the entrance and exit of hydrophilic low-molecular-weight substances. Porins have high immunogenicity as well as antigenicity, exposing epitopes on the bacterial surface. They are generally conserved in a bacterial species or even in bacterial families, and also share strong taxonomic associations in that they have high homology in both primary amino acid sequence and secondary structure (Jeanteur, Lakey and Pattus, 1991). Because of these properties, porins can be attractive vaccine candidates for heterologous immunity against Gram-negative bacterial infections (Rimler, 1996). Porin H is the major outer membrane protein in the envelope of *P. multocida* and conserved in several serotypes of *P. multocida* (Chevalier, Duclohier, Thomas, Shechter and Wroblewski, 1993). This protein exists as homotrimer in outer membrane, and the molecular weight of denatured monomer varies from 34 to 42 kDa (Chevalier *et al.*, 1993). The differences of the outer membrane protein H gene (*ompH*) by serogroups or serotypes in *P. multocida* are thought by the variety of the length and the sequence of the two variable regions in the *oomph* gene functioning as specific epitopes. The OmpH is a homologue of the P2 porin of *Haemophilus infuenzae* and a monoclonal antibody against OmpH could passively protect mice against infection (Vasfi *et al.*, 1997).

2.3. Diagnosis of HS

Clinical diagnosis of HS is based on a combination of clinical signs, gross pathological lesions, epidemiology and history of previous occurrence of HS in herd. Diagnostic tests for HS include culture and biological tests for

isolation of the organism, biochemical and serological tests (stereotyping) and molecular methods for strain differentiation within the serotype.

2.3.1. Isolation

Blood or blood swab from the heart or from jugular vein or from freshly dead case should be stored on ice until it reaches the laboratory. Blood collected from clinical cases prior to death is not reliable since septicaemia is a terminal event (Shigidi and Mustafa, 1979). A small volume (0.2 ml) of suspension is inoculated into mouse by S/C or I/M route. If HS organisms are present, the mice will die within 24 hours.

2.3.2. Microscopy

Characteristic bipolar staining of short bacilli of *pasterella multocida* organisms in blood or tissue smears stained with methylen blue or gram positive stain indicative infection with *P.multocida*.

2.3.3. Cultural Characteristic

Pure cultures of *P.multocida* can be obtained from collected samples streaked onto blood agar. Large, smooth, non mucoid grayish colonies 1-2 mm in diameter can be obtained if enriched media such as tryptose agar with yeast extract or casein-sucrose-yeast agar are used (Wijewardana, DeAlwis and Bashansz, 1986).

2.3.4. Serological tests (serotyping)

Confirmatory diagnosis is made by serotyping the cultured organism using rapid slide agglutination (Namioka and Murata, 1961) and indirect heamagglutination assay by Carter for capsular typing B2 or E2 biotypes (Carter 1955, 1961). Other tests include, agglutination test for somatic typing, agar gel immunodiffusion or counter immunoelectrophoresis assays for rapid identification of type B and E (Carter and Chengappa, 1980), tube agglutination test and the serum bactericidal test (Bain, 1963). Recently an

enzyme linked immuno-sorbent assay ELISA for identification of *P.multocida* was developed by Dawkins, Johnson, Spencer and Paten, (1990).

Somatic typing by agglutination test using HCL treated cells was employed by Namioka and Murata (1961) to determine type 6 somatic antigen which causes HS. Agar gel immunodiffusion using heat stable supernatant antigen to determine 2 and 5 somatic antigen was used by Heddleston, Gallagher and Rebers, (1972).

2.3.5. Molecular methods of diagnosis

Phenotypic identification of *P.multocida* based on biochemical reactions is often limited and usually only done on a species level, even though three subspecies are described. For molecular taxonomy and diagnostic purposes, a phylogenetic analysis of the three subspecies of *P.multocida* based on their 16S Rrna (rrs) gene sequence belongs to *P.multocida subsp. Septica* a distinguished branch on the phylogenetic tree of pasteurellaceae, due to a 1.5% divergence of its rrs gene compared to the other two, more closely related subspecies multocida and gallicida. This phylogenetic divergence can be used for the identification of *P.multocida subsp. Suptica* by rrs gene determination since they form a phylogenetically well isolated and defined group as shown with a set of feline isolates. The sequence based identification shows the advantages over conventional methods. It is therefore helpful for future unambiguous identification and molecular taxonomy of *P.multocida* as well as for epidemiological investigations (Kuhnert, Boerlin, Emler, Krawinkler and Frey, 2001). Molecular detection by PCR offers a more sensitive, specific, time-saving method for detecting pasteurella and confirming infection (Miflin and Blackall, 2001).

2.4. Immunity

The immune system protects organisms from infection with layered defenses of increasing specificity. Physical barriers prevent pathogens such as bacteria, fungi and viruses from entering the body. If a pathogen breaches these barriers, the innate immune system provides an immediate, but non-specific response. Innate immune systems are found in man and animals (Litman, Cannon and Dishaw, 2005). However, if pathogens successfully evade the innate response, vertebrates possess a third layer of protection, the adaptive immune system, which is activated by the innate response. Here, the immune system adapts its response during an infection to improve its recognition of the pathogen.

2.4.1. Innate immunity

Microorganisms that successfully enter a host will encounter the cells and mechanisms of the innate immune system of that host. The innate response is usually triggered when microbes are identified by pattern recognition receptors, which recognize components that are conserved among broad groups of microorganisms (Medzhitov, 2007). Innate immune defenses are non-specific, meaning these systems respond to pathogens in a generic way (Alberts, Johnson, Lewis, Raff, Roberts, and Walters, 2002). This system does not confer long-lasting immunity against a pathogen. The innate immune system is the dominant system of host defense in most organisms (Litman *et al*., 2005).

2.4.2. Adaptive immunity

The adaptive immune system evolved in early vertebrates and allows for a stronger immune response as well as immunological memory, where each pathogen is "remembered" by a signature antigen (Pancer and Cooper, 2006). The adaptive immune response is antigen-specific and requires the

recognition of specific “non-self” antigens during a process called antigen presentation. Antigen specificity allows for the generation of responses that are tailored to specific pathogens or pathogen-infected cells. The ability to mount these tailored responses is maintained in the body by "memory cells". Should a pathogen infect the body more than once, these specific memory cells are used to quickly eliminate it. B cells and T cells are the major types of lymphocytes and are derived from hematopoietic stem cells in the bone marrow. (Janeway, 2005). B cells are involved in the humoral immune response, whereas T cells are involved in cell-mediated immune response.

2.5. Immunity to *P.multocida*

The type of immunity developed against *P.multocida* is mainly antibody mediated. Lipopolysaccharide (LPS) of *P.multocida* stimulates humoral immunity, and it considered to be a protective antigen (Wijewardana and Sutherland, 1990). Nagy and Penn (1976) reported that antibody level in vaccinated cattle determined by passive mouse protection (PMP) test indicated that immunity to HS is exclusively antibody mediated. The duration and the magnitude of these responses are affected by many factors such as virulence, potency of the antigen, the dosage, and the method of induction and the age of the animal. *P.multocida* strains are classified into Heddleston serotypes based on the antibody responses to LPS, while antibodies raised against heat-killed *P.multocida* vaccines are directed primarily against LPS and protect the host against strains within the same serotype (Brogden and Rebers, 1978). LPS purified using the Westphal method and injected into mice and rabbits resulted in poor antibody response and no protection against *P.multocida* infection. In contrast, LPS injected into chickens induced a good antibody response which passively protected recipients against disease (Rebers, Phillips, Rimler, Boykins and Rhoades,

1980) In addition, an opsonic monoclonal antibody against LPS from a serotype B strain of *P.multocida* was shown to partialy protect mice against *P.multocida* infection (Ramdani and Adler, 1991) whereas monoclonal antibodies raised against the LPS from a serotype A strain were shown to be bactericidal and completely protect mice against homologous challenge (Wijewardana, Wilson, Gilmour and Poxton, 1990). LPS was found to stimulate TNF-α release from bovine alveolar macrophages and many other tumour inhibition factors and interleukins were released and triggered by the mitogenic action of the endotoxins. The mitogenic action was explained through the disturbance in the host cellular mechanisms, leading to cellular proliferation and blocking apoptosis (Horadagoda, Hodgson, Moon, Wijewardana and Eckersall, 2002).

2.5.1. Types of immunity to HS

Types of immunity to HS are recognized as naturally aquired, artifitial active or passively inducible.

2.5.1.1. Active natural immunity

In endemic areas, regular outbreaks of HS occur mostly during the wet season, depending on an interaction between a numbers of factors (burden of infection, host defense mechanism, immunity level). Some animals succumb to clinical disease while others will develop what is described as an arrested infection leading to a naturally acquired immunity (De Alwis, 1987). Thus, in endemic areas, the adult population through successive exposures acquires natural immunity and only small numbers of hitherto unexposed animals will remain susceptible at each outbreak. It is assumed that morbidity and mortality due to HS in a given population largely depend on the proportion of an immune to non-immune animals and the phenomenon of naturally acquired immunity is responsible for the

different patterns of morbidity and mortality in endemic and non endemic areas.

2.5.1.2. Active artificial immunity (Vaccination)

Vaccination is generally accepted as the best mean of control against pasteurellosis and vaccines in different forms are readily available throughout the world.

2.5.1.3. Passive artificial immunity

Mosier, Confer and Panciera (1989) reviewed the evolution of vaccines for bovine pneumonic pasteurellosis, where they stated that the use of HS antisera to induce passive immunity to HS was practiced since 1918. The use of HS antisera was found to be of value when given before shipment to control shipping fever.

2.6. Mucosal immunity

The mucosal surfaces of the gastrointestinal and respiratory tracts represent the principal portals of entry for most pathogens. The mucosal surfaces represent a critical component of the mammalian immunologic repertoire. The major antibody isotype in external secretions is secretory immunoglobulin A (S-IgA). Approximately 40 mg of IgA per kg of body weight is secreted daily, especially from the gastrointestinal tract, and the total amount of IgA synthesized is almost twice the amount of IgG produced daily in humans (Conley and Delacroix, 1987). It is, however, interesting that the major effecter cells in the mucosal surfaces are not IgA B cells, but T lymphocytes of $CD4^+$ as well as $CD8^+$ phenotypes. It is estimated that T lymphocytes may represent up to 80% of the entire mucosal lymphoid cell population.

2.7. Common mucosal immunology

Mucosal inductive sites such as the Peyers patches in the intestinal tract and the nasal-associated lymphoreticular tissue in the oropharyngeal cavity, stand as sentinels to the intestinal and respiratory tracts and represent the major sites where mucosal immune responses are initiated (Figure**:** 1:1). Common features of these inductive sites are microfold although the precise function of M cells has not yet been established; recent studies indicate that they are involved in the uptake, transport, processing, and possibly presentation of microbial antigens (Neutra, Frey and Kraehenbuhl, 1996). The interaction of epithelial cells with T and B lymphocytes induces epithelial cells to differentiate into M cells *in vitro*, indicating the importance of lymphocyte-epithelial cell interactions for maintaining M cells in the follicle-associated epithelium of the Peyer's patches (Jones, Ghori and Falkow, 1994). These lymphocyte-M cell interactions can occur in the pocket of the M cells and are mediated through thin cellular extensions, indicating that cell-cell interactions are an intricate part of the M-cell function and that they may facilitate transfer of luminal antigens.

2.7.1. T-Cell and Cytokine Involvement in B-Cell Isotype Commitment to IgA

Although the variables involved in the switching of B cells to polymeric IgA (pIgA) producing plasma cells have been studied, many questions remain. In recent years, gene-deleted or knockout mice have contributed to a better understanding of the role of specific cells, cytokines, and surface molecules involved in IgA isotype switching. Presumably, isotype switching occurs in mucosal inductive sites, while IgA production by plasma cells occurs in mucosal effector sites, separating the IgA switching and IgA secretion by B cells into different immune compartments (McGhee,

Lamm and Strober, 1999). Each of these stages requires specific signals, such as co stimulatory molecules, cytokines, and T-helper cells, to give rise to antigen specific S-IgA Abs in mucosal effector sites. Neither Th1- nor Th2-type cytokines contribute significantly to the switching of B cells to surface IgA positive (sIgA+) B cells (Figure**:** 1: 2). This process requires the presence of transforming growth factor ßð1 (TGF-ßð1), which can activate the switching of B cells to the IgA isotype (Ehrhardt, Strober and Harriman, 1992). TGF-ßð1 induces a small proportion (<2%) of B cells to switch to IgA in activated B-cell cultures (Ehrhardt *et al*., 1992). However, TGF- ßð1, when used in combination with additional signals, increased TGF-ßð1-induced switching in 10% to 20% of B cells and approached IgA+ B-cell levels observed in Peyer's patches (McIntyre, Kehry and Snapper, 1995). Thus, multiple activation signals contribute to the switch to IgA, i.e., B-cell activation by cross-linking the B-cell antigen receptor, CD40-CD40L interactions to promote switching, TGF-ßð1 by directing the switch to IgA, and Th2-type cytokines by increasing the number of post-switch IgA+ B cells and their differentiation into IgA-secreting plasma cells. In addition, activated T cells and dendritic cells from the Peyer's patches are more effective in switching sIgM+ sIgA- B cells to IgA-producing cells than were T cells and dendritic cells derived from the spleen (Spalding, Williamson, Koopman and McGhee, 1984). This suggests that mucosal inductive sites contain specialized T cells or dendritic cells beneficial for B cells to differentiate into IgA-producing cells. Thus, either Th1 or Th2 cells or a combination of these cell types can support antigen-specific S-IgA Ab responses. In this respect, Th2-type cytokines play a role in terminal differentiation of B cells, that are already committed to IgA (Briere, Bridon, Chevet, Souillet, Bienvenu and Guvet, 1994), while the Th1- type cytokine

IFN-γð has been implicated in the induction of the polymeric Ig receptor (pIgR needed for transport of S-IgA (Wira, Richardson and Prabhala, 1994). Cross inhibition of Th1 and Th2 cell-directed IgG2a and IgE production was mediated through IFN-γð and IL-4, respectively (Figure 1:2) (Rizzo, DeKruyff, Umetsu and Caspi, 1995).

2.7.2. Mucosal S-IgA Antibody Responses

The hallmark of the mucosal immune system is the production of S-IgA. S-IgA results from transcytosis of dimeric IgA across the epithelium through binding to the pIgR. S-IgA is released from the pIgR by cleavage of the receptor, resulting in pIgA covalently associated with a substantial part of the pIgR, i.e., the secretory component (Mostov, 1994). This complex, referred to as S-IgA, seems to be more resistant to proteolysis in external secretions. Furthermore, transport of pIgA containing immune complexes across epithelial cells expressing the pIgR is another defense mechanism of the mucosal immune system against pathogen entry (Kaetzel, Robinson and Lamm, 1994). These characteristics of pIgA are beneficial in preventing infection and inflammation at epithelial surfaces.

2.8. Common Mucosal Immune System

The immunologic network operating on external mucosal surfaces consists of gut-associated lymphoid tissue (GALT), the lymphoid structures associated with bronchoepithelium and lower respiratory tract (BALT), occular tissue, upper airway, salivary glands, tonsils and nasopharynx (NALT), larynx (LALT), middle ear cavity, male and female genital tracts, mammary glands, and the products of lactation . The organized lymphoid follicles in the GALT and BALT are considered the principal inductive sites of mucosal immune response (Staats, Jackson, Marinaro, Takahashi, Kiyono and McGhee, 1994).

2.8.1. Gut-associated lymphoid tissue (GALT) and Bronchoepithelium associated lymphoid tissue (BALT)

A substantial body of information has been generated with Peyer's patches and other organized lymphoid follicles in the GALT, including the appendix (Dasso and Howell, 1997), and the BALT concerning the induction of mucosal immune responses and the development of systemic hyporesponsiveness following oral exposure to an antigen (oral tolerance) (Javed, Gienapp, Cox and Whitacre, 1995).

The common features of all inductive mucosal sites include an epithelial surface containing M cells (cells with microfold) overlying organized lymphoid follicles. Mucosal epithelium is a unique structure, and in addition to M cells, it contains mucin-producing glandular cells, lymphocytes, plasma cells, dendritic cells, and macrophages. The mucosal epithelial cells express polymeric immunoglobulin receptor (pIgR) and secretory component, major histocompatibility complex (MHC) class I and II molecules, other adhesion molecules, and a variety of cytokines and chemokines (McGhee *et al*., 1999).

The dendritic cells are present in different components of the common mucosal immune system, including the organized lymphoid tissue and the mucosal epithelium. These cells can be strongly associated with potentiation of immune response and promote development of active immunity (Liu and MacPherson, 1993). Recent observations have suggested that dendritic cells are potent antigen-presenting cells (APC) and are critical in initiating primary immune responses, graft rejection, autoimmune disease, and generation of T-cell-dependent B-cell responses. The APC function is attributed in part to their ability to express co stimulatory molecules (CD80

and CD86) and other accessory ligands necessary for up regulation or induction of tolerance (Steinbrink, Wölfl, Jonuleit, Knop and Enk, 1997).

2.8.2. Nasopharynx-associated lymphoid tissue (NALT).

Recent studies in the rat, mouse, and hamster have shown the presence of organized lymphoid tissue at the entrance of the nasopharyngeal duct. This represents an important component of mucosal lymphoid tissue in the rodents (Small, Smith and Moss, 1985). The NALT appears to have better-developed lymphoid follicles, with marked intraepithelial infiltration by lymphocytes. The follicular areas are organized into B cells and intrafollicular (T-cell) areas of approximately similar size. The rodent NALT contains a wealth of dendritic cells. The lymphoid follicles are covered by ciliated epithelium containing few goblet cells and numerous M cells. The NALT M cells appear to be identical to those in Peyer's patches and BALT and are involved in similar immunological functions, including antigen uptake and subsequent mucosal immune responses to specific antigens (Sminia and Kraal, 1999). In humans, the nasopharyngeal lymphoid tissue is represented by the salivary glands and other glandular tissue in the Waldeyer's ring, which consists of paired palatine and tubal tonsils and unpaired pharyngeal and lingual tonsils. It is not clear how comparable the functional role of Waldeyer's ring in humans is to the NALT in rodents. However, there is increasing evidence to suggest that human tonsillar and adenoidal tissues are important components of mucosal immunity and function in a manner similar to those of GALT or BALT (Bernstein, Gorfien and Brandtzaeg, 1999). The tonsils consist of several lymphoid elements. These include follicular germinal centers, mantle zones of lymphoid follicles, the extrafollicular areas, and the reticular crypt epithelium on the surface in constant contact with the external environment. The tonsillar

epithelium contains a significant number of dendritic cells, M cells, memory B cells, and scattered B and T cells. The formation of the germinal center takes place shortly after birth, secondary to the activation by environmental antigens, and plasma cells appear in tonsils by 2 to 3 weeks of age. Unlike Peyer's patches, tonsils exhibit considerable *in situ* arise during T-cell-dependent B-cell responses) generate plasma blasts and plasma cells of both IgG and IgA isotypes.

There is, however, a predominance of IgG isotype (60 to 70% IgG versus 15 to 20% IgA). The follicular germinal centers are often associated with clonal expansion of B cells, somatic hyper mutation in the B-cell immunoglobulin variable-region gene, positive selection of B cells, and eventual B-cell differentiation to memory cells and isotype-specific plasma cells (Bernstein *et al.*, 1999).The tonsils, nasal and bronchial mucosa, and salivary glands exhibit similar distribution of IgA and IgD immunocytes. In addition, scattered areas in the crypt epithelium of nasopharyngeal tonsils (but not palatine tonsils) express secretary component. Another important feature of mucosal lymphoid tissue and the follicular germinal center is induction of the J-chain gene in some B-cell subsets. Tonsillar germinal centers express a very high percentage of extra follicular immunocytes with J-chain expression. More than 90% of these immunocytes are of the IgA isotype (Bernstein *et al.*, 1999).

2.9. Vaccination

Long-term active memory is acquired following infection by activation of B and T cells. Active immunity can also be generated artificially, through vaccination. The principle behind vaccination is to stimulate the immune system and develop specific immunity against that particular pathogen without causing disease associated with that organism (Alberts *et al.*, 2002).

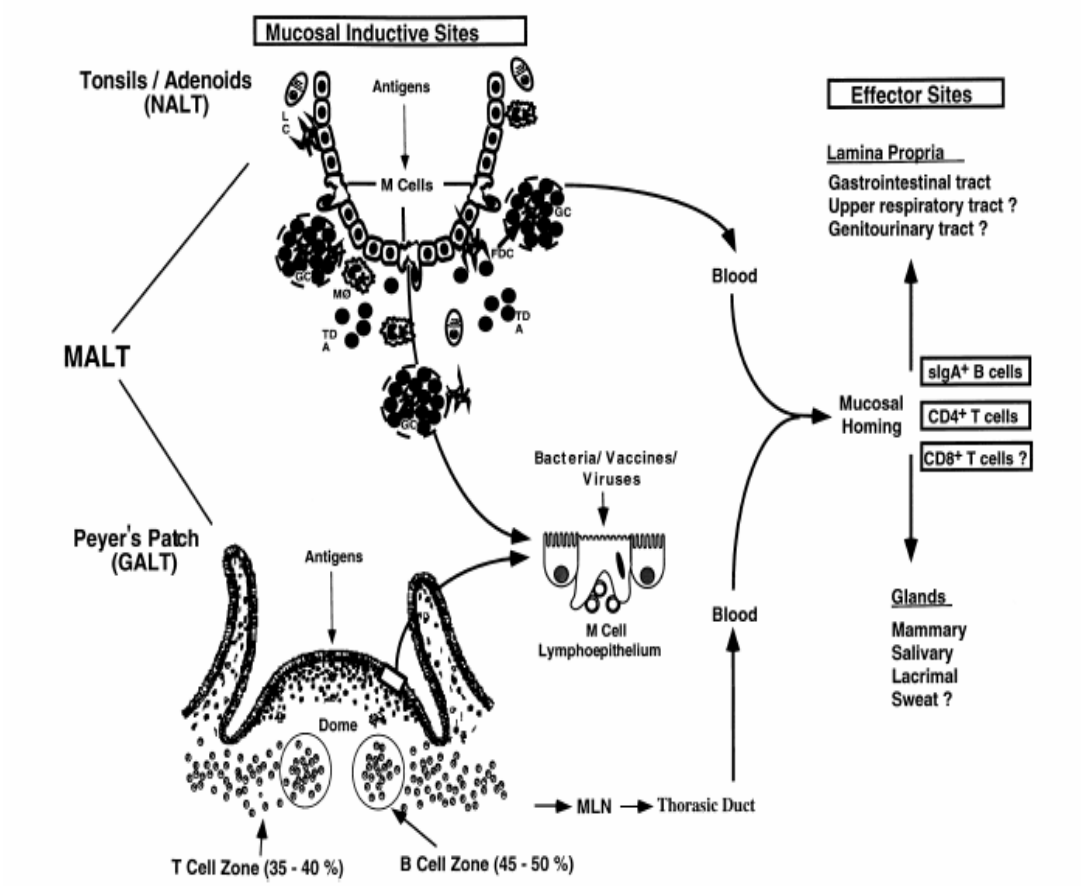


Figure (1.1): M cells and the induction of mucosal immunity.

M cells are present in mucosal inductive sites in both the intestinal and upper respiratory tract, specifically in Peyer's patches and the nasal-associated lymphoid tissue, the tonsils and adenoids. M cells are thought to play an important role in antigen processing and possibly the induction of antigen-specific mucosal immunity in mucosal effector sites. Tissues followed by question marks are presumed sites since limited data are available on these tissues.

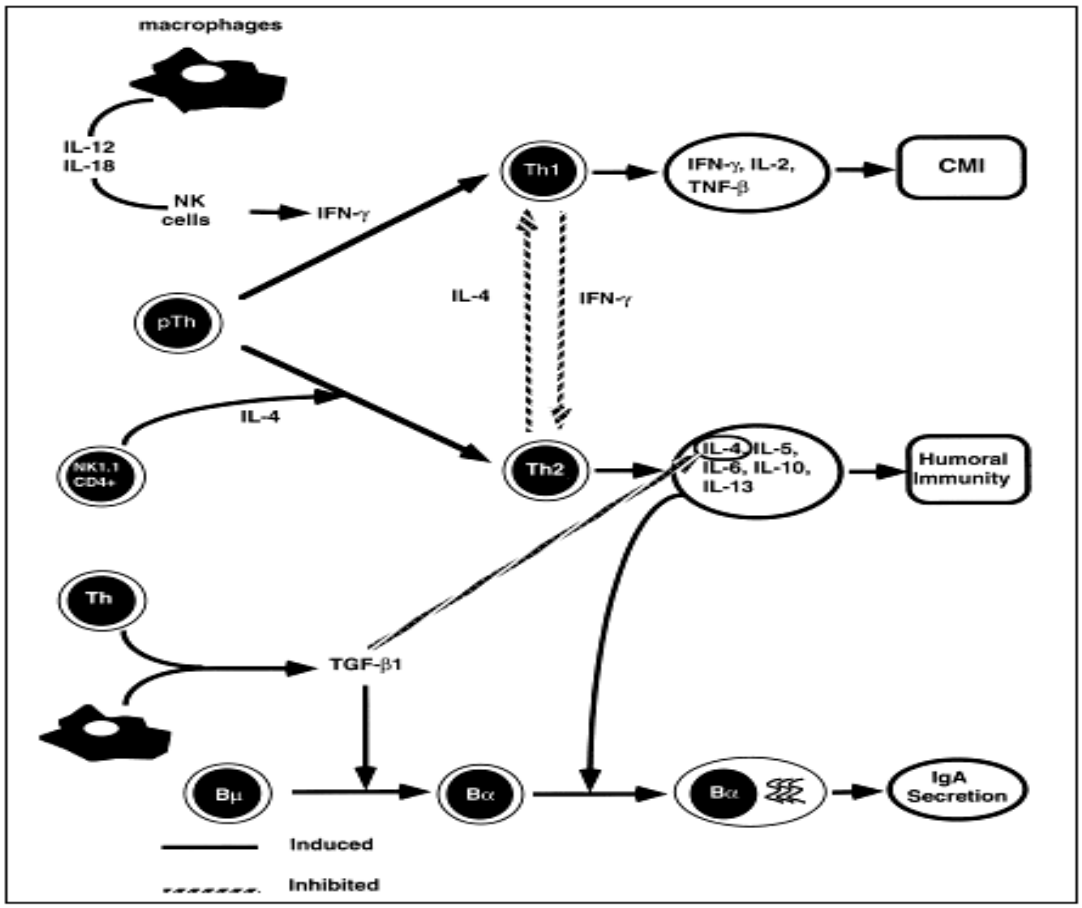


Figure (1.2): Differentiation and regulation of T-helper subsets and the immune response in the mucosal compartments.

Encounter of pathogen- derived antigen or vaccine antigen will stimulate T-helper cells to secrete cytokines. Depending on the stimulus, a Th1 or Th2 cell response is induced. For example, intracellular pathogens will induce production of IL-12/IL-18 by macrophages, activating IFN-γð production by NK cells and inducing differentiation to a Th1- mediated immune response, which supports CMI and production of complement-fixing antibodies, presumably by production of cytokines such as IFN-γð, IL-2, and TNF-γð. A Th2 response can be observed upon infection with parasites or upon vaccine administration; this response is characterized by reduction of cytokines such as IL-4, IL-5, IL-6, IL- 10 and IL-13 which support humoral immunity. However, for induction of an S-IgA, TGF- ß ð1 is required to enable B-cells to switch to IgA. TGF- ßð1 production is associated with inhibition of IL-4 production by Th2 cells inhibiting IgE production.

This deliberate induction of an immune response is successful because it exploits the natural specificity of the immune system, as well as its inducibility. With infectious diseases being the leading causes of death in the human population, vaccination represents the most effective manipulation of the immune system mankind has developed, (Janeway, 2005). Vaccine is a product which may be suspension of living or dead cells ,or an antigenic fraction of cells, which when injected with or without adjuvant enhances the immunity production in the animal's body (Bain, 1954).

2.9.1. Mucosal vaccination

The primary reason for using a mucosal route of immunization is that most infections affect or initiate the infectious process at the mucosal surfaces, and that in this infection, mucosal application of a vaccine is often required to induce a protective immune response (Holmgren and Czerkinsky, 2005). Advantage of mucosal vaccination is that this mode can induce both mucosal and systemic immune responses, which results in two layers of host protection against infectious diseases (O'Hagan, MacKichan and Singh, 2001). An effectively designed mucosal vaccine must: (1) protect from physical elimination and enzymatic digestion, (2) target mucosal inductive tissues including M cells, and (3) appropriately stimulate the innate immune system to generate effective adaptive immunity. In mucosal vaccine development, it is crucial to select the appropriate immunization route, and most current mucosal vaccine delivery is intended to mimic the natural encounter of mucosal inductive sites with environmental antigens and pathogens. Mucosal vaccination is usually performed by either oral (enteric) or intranasal (respiratory tract) application. In fact, many new vaccines are being tested by both routes to determine which induces immune responses via the CMIS most effectively. In many cases, intranasal

immunization is more effective and in general requires smaller vaccine doses with less adjuvant. Intranasal immunization has been shown to result in the development of antibody response in the mammary glands and in the genital tract (Conley and Delacroix**,** 1987). The occasional inefficiency of oral immunization is due in part to degradation of vaccine by the acidic gastric pH and proteolytic enzymes as well as by the potential for the induction of mucosally induced tolerance. In addition, a recent study by Glenn, (2000) provides a strong evidence that transcutaneous immunization is a simple and practical innovation that may improve the vaccine delivery.

Mucosal immunization induces antigen-specific Th1- and/or Th2-type responses depending on the nature of the antigen, adjuvant and antigen delivery vehicle used. For example, administration of intracellular pathogens such as *Salmonella* led to the formation of Th1 cells producing IFN-γ, IL-2 and TNF-β (Hess, Ladel, Miko and Kaufmann, 1996). Murine Th1-type immune responses are associated with the development of CMI and B-cell responses characterized by IgG2a synthesis. On the other hand, the use of cholera toxin (CT) as a mucosal adjuvant with soluble protein antigen induces antigen-specific Th2 cells producing IL-4, IL-5, IL-6, and IL-10. Among these Th2-type cytokines, IL-4 has been shown to switch μ heavy chain to certain γ subclasses (e.g., IgG1) and to e subclasses (e.g., IgE) (Rousset, Garcia, and Banchereau, 1991). Furthermore, Th2 type cells are considered to be the major helper phenotype for the support of IgA responses via the production of IL-5 and IL-6 in the mouse system.

2.9.2. Types of vaccines

2.9.2.1. Killed vaccines

Are vaccines containing killed microorganisms. These are previously virulent micro-organisms that have been killed with chemicals or heat

(Wakefield, Murch, Anthony, Linnell, Casson, Malik, Berelowitz, Dhillon, Thomson, Harvey, Valentine, Davies and Walker-Smith, 1998). The antibody response against killed vaccines depends on the method of cultivation, way of preparation and killing, presence of adjuvants, degree of antigenic variation, route of administration and dose.

2.9.2.2. Attenuated vaccines

Attenuated live vaccines are prepared by passaging the organism in non–ordinary host or by growing it in unfavorable conditions. Attenuation trials of HS vaccine were achieved by passage in pigeons .The use of live attenuated pasteurella organisms as vaccine to prevent the disease caused by *p.multocida* was reported by Bierer, (1969).

2.9.2.3. Toxoid vaccines

A toxoid is an inactivated toxin, the harmful substance produced by a microbe. Toxoid vaccine was proved useful against atrophic rhinitis caused or associated with toxigenic strain of *P.multocida* (Bording and Riising, 1986).

2.10. Adjuvants

The word adjuvant is derived from the Latin word adjuvare, which means help or aid or to enhance (Vogel, 1998). Singh and O'Hagan (2003) stated that immunological adjuvants were originally described by Ramon in 1924 as "substances used in combination with a specific antigen that produced a more robust immune response than the antigen alone". This broad definition encompasses a very wide range of materials (Vogel and Powell, 1995). The first adjuvants were developed in the 1920's and a number of substances including chemicals, microbial components and mammalian proteins have been used to boost the immunity (Cox and Coulter, 1992; 1997).

Adjuvants are structurally heterogeneous compounds that enhance or modulate the immunogenicity of the poorly immunogenic vaccine proteins or peptides (Gupta, Relyveld, Lindblad, Bizzini, Ben-Efraim and Gupta, 1993). Most vaccines traditionally consist of live attenuated pathogens, whole inactivated/killed organisms or inactivated toxins containing many immunopotentiators essential for activating integrated protective immune responses. An adjuvant can be used for increasing the immunogenicity of poor antigen, improving the efficacy of vaccine in new born and reducing the amount of antigen or the number of immunizations (McElrath, 1995). Compared to injection of antigen alone, injection of antigen plus an adjuvant generally permits use of a much smaller quantity of the antigen and greatly enhances the antibody titer (Kaeberle, 1986).

2.10.1. Roles of adjuvants in modulation of immune responses

Adjuvants can be used to improve the immune response to vaccine antigens for several different purposes, including: (1) increasing the immunogenicity of weak antigens; (2) enhancing the speed and duration of the immune response; (3) modulating antibody avidity, specificity, isotype or subclass distribution; (4) stimulating cell mediated immunity; (5) promoting the induction of mucosal immunity; (6) enhancing immune responses in immunologically immature or senescent individuals; (7) decreasing the dose of antigen in the vaccine to reduce costs or (8) helping to overcome antigen competition in combination vaccines (Singh and O'Hagan, 2003). Adjuvants were initially thought of as agents capable of promoting and sustaining antibody response. However, new evidence has shown that adjuvants influence the titer, duration, isotype and avidity of antibody, and affect the properties of cell-mediated immunity (Hunter, Olsen and Bennett, 1995).

2.10.2. Classification of adjuvants

Different scientists used different criteria for classifying the adjuvants. Cox and Coulter (1992) classified adjuvants into particulate and non-particulate groups. According to Vogel (1998) adjuvants can be classified according to their sources, mechanism of action or physiochemical properties. (Edelman, 1997; reviewed by Allison and Byars (1991)) classified adjuvants into three groups: (1) immunostimulatory adjuvants, (2) carrier adjuvants and (3) vehicle adjuvants. Adjuvants are also classified: (1) according to their route of administration like mucosal or parental routes; (2) divided into alum salts and other mineral adjuvants, tensoactive agents, bacterial derivatives, vehicles and slow release materials or cytokines (Byars and Allison, 1990). (3) Divided into the groups: gel-based adjuvants, tensoactive agents, bacterial products, oil emulsions, particulate adjuvants, fusion proteins or lipopeptides (Jennings, Simms and Heath, 1998).

Mostly aluminum or oil adjuvants are used in vaccine, but these chemical adjuvants that have many disadvantages, such as side effects, strong local stimulation and carcinogenesis, together with complicated preparations or failure to increase immunogenicity of weak antigen, (Bowersock and Martin, 1999). Comparative studies in humans and animals showed that aluminum is a weak adjuvant for antibody induction to recombinant protein vaccines and induces a Th2, rather than a Th1 response (Gupta, 1998). Some other groups of adjuvants including oil emulsions, lipopolysaccharides, polymers, saponins, liposomes, cytokines, ISCOMs, Freund's complete adjuvant, Freund's incomplete adjuvant, alums, bacterial toxins etc., have been evaluated, although the mechanism of action of adjuvants often remain poorly understood (Edelman, 1997).

2.10.3. Mucosal Adjuvants

Adjuvant has a complex mechanism of action as an immunostmulator, delaying the absorption of vaccine and prolonging the antigenic stimulus to the antibody forming cells. A number of chemical agents have been tested to enhance the immunogenicity of mucosally administered antigens (especially for nonreplicating antigens), including cholera toxin (CT) holotoxin (B subunit), *E. coli* heat-labile toxin, lectins and polyelectrolytes, ISCOM, actins, avridine, low-oil emulsion (MF59), lipid A, lysophosphatidyl glycerol, and cytokines (IL-5). Such antigens in general induce only weak or insufficient immune responses when administered mucosally. Adjuvants influence virtually every aspect of immune response to an antigen (Elson and Dertzbaugh, 1999).

2.10.3.1. Olive Oil

Olive oil contains triacylglycerols and small quantities of free fatty acids, glycerol, pigments, aroma compounds, sterols, tocopherols, phenols, unidentified resinous components (Kiritsakis, 1998)

2.10.3.1.1. Oleic acid

Olive oil contains a high percentage of monounsaturated oleic acid. Thus, it is natural monounsaturated oil. This particular fatty acid reduces LDL-cholesterol, which is responsible for the formation of the atherosclerotic plaque, and increases the HD-cholesterol. Hydrocarbon squalene: The hydrocarbon squalene, an important metabolic factor, is present in high percentages in olive oil.

2.10.3.1.2. Phenolic compounds

Olive fruit contains simple and complex phenolic compounds. Most of these compounds pass into the oil increase its oxidative ability and improve its taste. Hydrohytyrosol, tyrosol and some phenolic acids are mainly found

in olive oil (Kiritsakis, 1998). The phenol content and the specific composition of these phenols in olive oil depend on the altitude where the olive trees are grown, on the harvesting time and on the processing conditions (Cinquanta, Esti and Notte, 1997; Kiritsakis, 1998).

2.10.3.1.3. Tocopherols

Olive oil contains the tocopherols α-, β-, γ-, δ- (α-tocopherol covers almost 88%). The tocopherol content of olive oil depends not only on the presence of these compounds in olive fruit but also on several other factors involved in the transportation, storage and olive fruit processing. According to Viola (1997), the ratio of vitamin-E to polyunsaturated fatty acids in olive oil is better than to other edible oils.

2.10.3.2. Sesame oil

Sesame (*Sesamum indicum* L., Pedaliaceae) is a very old cultivated crop and thought to have originated in Africa (Ram, Catlin, Romero and Cowley 1990). Sesame oil is fixed oil obtained by expression from the seed of Sesamum indicum L. It is a pale-yellow liquid with a slight, pleasant odour and a bland taste, it does not solidify when cooled to 0 °C. Sesame oil consists of glycerides, fatty acids such as oleic and linoleic acids, with small proportions of stearic, palmitic and arachidic acids. It also contains about 1% of sesamin, and a smaller amount of sesamolin. Sesame oil is slightly soluble in alcohol, miscible with ether, chloroform and light petroleum. And can be sterilized by dry heat. Chlorosesamone obtained from roots of sesame has antifungal activity (Begum, Furumot and Fukui, 2000). Sesame lignans have antioxidant and health promoting activities. High amounts of both sesamin and sesamolin have been identified in sesame (Sirato-Yasumoto, Katsuta, Okuyama, Takahashi and Ide, 2001). Both sesamin and sesamolin were reported to increase both the hepatic mitochondrial and the

peroxisomal fatty acid oxidation rate. Sesame seed consumption appears to increase plasma gamma-tocopherol and enhanced vitamin E activity which is believed to prevent cancer and heart disease (Cooney, Custer, Okinaka and Franke, 2001). Sesame has an antibacterial effect as was proven by Acar and Goldstein (1991), using the disk diffusion method. Sesame oil is a pharmaceutic aid used as a solvent for intramuscular injections (Tyler, Brady and Robbers, 1976). The oil was used during the 4th century by the Chinese as a remedy for toothaches and gum disease. Sesame oil is known to reduce cholesterol due to the high polyunsaturated fat content in it. Other uses of sesame oil include the treatment of blurred vision, dizziness, and headaches. The Indians have used sesame oil as an antibacterial mouthwash (Annussek, 2001). A recent clinical trial proved that sesame oil was significantly effective for treating dryness of nasal mucosa (Johnson, Bratt, Michel-Barron, Glennow and Petruson, 2001).

2.10.3.3. Vitamin D3 (Vit D3)

It was first demonstrated in mice by Enioutina, Visic and Daynes, (2000) that an intramuscular, subcutaneous or intradermal immunization with a microbial antigen and aluminium hydroxide as an adjuvant induces a mucosal immune response when 1α, 25(OH) 2D3 (Vit D3, calcitriol), the active metabolite of vitamin D, is added as an additional immunomodulator. Mice showed enhanced antigen-specific IgG and IgA in serum and all tested mucosal secretions (tears, oral, vaginal and colorectal secretions) (Daynes, Enioutina, Butler, Mu, McGee and Araneo, 1996) and increased numbers of IgA and IgG secreting cells (ASC) in systemic (the local draining lymph node and spleen) and mucosa-associated lymphoid tissues (Peyer's patches, mesenteric lymph nodes). Vit D3 modulated the production of cytokines in the local draining lymph node. When cells from the local draining lymph

node of a mouse treated with Vit D3 were stimulated in vitro with anti-CD3€ an enhanced production of IL-4, IL-5 and IL-10 (Th2-cytokines) and a reduced production of IL-2 and IFN-γ Th1-cytokines) was seen (Daynes *et al*, 1996). So, in the local draining lymph node, a switch towards a Th2-cytokine profile occurred, as is necessary for an IgA response. Furthermore, migration of antigen- pulsed dendritic cells from the local draining lymph nodes towards the Peyer's patches was seen, where the activation and differentiation of antigen-specific B cells for a mucosal immune response is initiated (Enioutina *et al*., 2000). Vit D3 exerts its effects on the immune system by binding to a nuclear receptor (nVDR), which is present in activated Th, cytotoxic T cells (CTL) as well as in activated B cells (Baran and Sorensen, 1994) via this receptor, Vit D3 modulates the production of cytokines (Lemire, Adam, Sakai and Jordan, 1984). By reducing the transcription and secretion of among others IFN-γ, IL-2, IL-8, IL-12 and GM-CSF. IL-12 is the most important cytokine for promoting differentiation of Th0 cells towards Th1 cells. Furthermore, Vit D3 favours the induction of D4+CD25+ regulatory T cells and enhances the production of Th2 cytokines such as IL-4 and IL-10, which in turn inhibits Th1 responses. Vit D3 is also known to stimulate TGF-ß that is involved in mucosal immunity, isotype switching towards IgA as well as IgG2b.

CHAPTER TWO
MATERIALS AND METHODS

3.1. Haemorrhagic septicemias (HS) vaccine

HS vaccine was donated by the Central Veterinary Research Laboratories, Soba, (without adjuvant). Vaccine was produced in the Gottingen bioreactor (fermenter) under the optimized physical growth condition temperature at 37ºC, pH 7.4 and stirring ×300 rpm and contained 10^9 cfu/ml. All production steps of the vaccine were performed under strict aseptic condition, as was reported by Elbashir, (1993).

3.2. Preparation of whole cell lysate

The Isolate of *P.multocida* 222 sero type B6 was obtained from the freeze-dried stock of the Department of Microbiology, Faculty of Veterinary Medicine, University of Khartoum. The isolate was reconstituted in nutrient broth and incubated at 37°C for 24 hours. Growth was checked and tested for purity and the culture was kept in cooked meat medium for preparation of whole cell lysate.

Pasteurella multocida serotype B 6 was grown in nutrient broth for 48 hour at 37°C. One liter culture was harvested by centrifugation at ×4000 rpm for 20 minutes at 4°C (Megafuge 1.0R, Germany) and the packed cellular mass was washed three times with phosphate buffer saline pH 7.2. The cells were then resuspended in the same buffer and subjected to ultrasonic disruption (sonnicator) (MSE-England) at ×18000rpm for 10 minutes at short intervals at 4°C (Mortenson, Valentine and Carnhan, 1962). Cell debris and unbroken cells were removed by centrifugation at 3000 rpm for 15 minutes and the supernatant was stored frozen until used.

3.3. Laboratory animals

3.3.1. Rats

Forty eight rats were kept in conventional animal house at the Department of Microbiology, allowed to adapt to food and water and later divided into five groups (1-5). Groups 1, 2 and 3 were further divided into sub groups (a) and (b) each containing 6 rats. Groups 4 and 5 were control groups of 6 rats each.

3.4. Bacteriological media

Are prepared according to the manufacturer's recommendations.

3.4.1. Blood agar

This medium is composed of nutrient agar, which consists of:

Lab-lemco powder	1 g
Yeast extracts	2 g
Peptone	5 g
Sodium chloride	5 g
Agar	15 g

Preparation

One liter was prepared as described by Barrow and Feltham (2003). Forty grams of blood agar base No.2 were suspended in 950 ml of distilled water, pH was adjusted to 7.4 and then sterilized by autoclaving at 121°C for 15 minutes. Fifty milliliters of defibrinated blood were added after cooling to 50°C and mixed. The complete medium was poured into sterile Petri dishes and left to solidify at room temperature on leveled surface.

3.4.2. Nutrient broth

Lab-lemco powder	1 g
Yeast extracts	2 g
Peptone	5 g

Sodium chloride	5 g

Preparation

It was prepared according to Barrow and Feltham (2003) by dissolving 13 grams of the medium in one liter of distilled water. The pH of the medium was adjusted to 7.4 and was distributed into screw capped bottles 5 ml each and sterilized by autoclaving at121°C for 15 minutes.

3.4.3. Robertson's cooked meat medium

The medium was prepared according to Barrow and Feltham (2003). It consists of 1000 grams of minced meat and 1000 ml of o.o5 N-NaOH. The minced meat was added to the alkali solution, mixed well then heated to boiling and simmered for 20 minutes with frequent stirring. The fat was skimmed off and the pH was adjusted to 7.5. The liquid part was strained through muslin and excess liquid was squeezed out.

Meat particles were dried at a temperature below 50°C, then sufficient dried meat was placed in screw capped container to depth of about 2.5 cm. A sufficient nutrient broth was added then sterilized by autoclave at 115°C for 20 minutes.

3.5. Solutions

3.5.1. Phosphate buffer saline (PBS)

Sodium chloride	8 g
Potassium chloride	0.2g
Disoduim hydrogen phosphate	1.15 g
Potassium dihydrogen phosphate	0.2 g

Preparation

These buffers were dissolved at 1liter of distilled water, pH 7.2 and autoclaved for 15 minutes at 121°C and allowed to cool at room temperature.

3.5.2. Normal saline

It was prepared by dissolving 8.5g NaCl in one liter of distilled water and autoclaving at 121°C for 15 minutes.

3.5.3. Borate-succinate buffer

Consist of solution A (Sodium tetraborate (19.0g/l)), solution B (succinic acid (5.9g/l)), Sodium chloride and horse serum. Solution B was added to one liter of solution A until pH 7.5 was reached. Then 0.14 NaCl and heat inactivated 1% horse serum were added.

3.5.4. Alsever's solution

Glucose	20 g
Sodium citrate	4.2 g
Trisodium citrate	8.0 g
Citric acid	0.55 g
Distilled water	1000 ml

Preparation

These were mixed and autoclaved at 115°C or 15 min.

3.6. Mucosal adjuvants

Olive oil, sesame oil were included as mucosal adjuvants in this study whereas vitamin D3 was used as safe mucosal adjuvant as was reported by (Daynes, *et al.*, 1996).

3.6.1. Preparation of Mucosal adjuvant vaccine

The national HS vaccine was mixed with each mucosal adjuvant in the ratio: 1:1 to contain 5×10^8 cfu/ml and the ratio 2:1 to contain 3.3×10^8 cfu/ml. The preparation was administered intranasally and all rats received a booster dose on day 15 post initial inoculation. A control group received phosphate buffered saline and another control group received vaccine alone, as is shown in table (2.1).

3.7. Immunization of rats

3.7.1. Group 1

In this group, olive oil was used as mucosal adjuvant. Rats were divided into two sub groups, G1 (a) and G1 (b), each consisted of 6 rats. G1 (a) were immunized intranasaly with 30µl of 1:1 vaccine adjuvant mixture and boostered with 20µl 2 weeks post-initial vaccination. Rats in G1 (b) were immunized intranasaly with 30µl of 2:1 vaccine adjuvant mixture and boostered with 20µl of the same preparation at day 15 post-initial vaccination.

3.7.2. Group 2

In this group, sesame oil was used as mucosal adjuvant. Rats were divided into two subgroups, G2 (a) and G2 (b), each consisted of 6 rats. G2 (a) were immunized intranasaly with 30µl of 1:1 vaccine adjuvant mixture and boostered with 20µl 2weeks post-initial vaccination. Rats in G2 (b) were immunized intranasaly with 30µl of 2:1 vaccine adjuvant mixture and boostered with 20µl of the same preparation at day 15 post-initial vaccination.

3.7.3. Group 3

In this group, vitamin D3 was used as mucosal adjuvant. Rats were divided into two sub groups, G3 (a) and G3 (b), each consisted of 6 rats. G3 (a) were immunized intranasaly with 30µl of 1:1 vaccine adjuvant mixture and boostered with 20µl 2 weeks post-initial vaccination. Rats in G3 (b) were immunized intranasaly with 30µl of 2:1 vaccine adjuvant mixture and boostered with 20µl of the same preparation at day 15 post-initial vaccination.

3.7.4. Group 4

In this group, six rats were immunized intranasaly with 30µl of the national Hemorrhagic septicemia (HS) vaccine without adjuvant and boostered intranasaly with 20µl of the vaccine 2 week after the initial dose.

3.7.5. Group 5

In this group, six rats were immunized intranasaly with 30µl of sterile Phosphate buffered saline (PBS) alone, boostered intranasaly with 20µl of the sterile PBS on day 15.

3.8. Tracheobronchial Lavage

Rats in each group were killed one week after second immunization ie on day 21. Animals were dissected, respiratory tracts (lung, trachea) were removed, and the trachea and bronchial tree were washed with 5ml PBS each in petri-dishes. The respiratory-lavage was transferred into test tubes and centerifugated at ×1000 rpm (MSE, BRITAIN) for 15 minutes the supernatants were collected and kept at -20 ºC until used.

3.9. Serological tests

The immunodiffution test and passive haemagglutination test were used to determine the presence of *P.multocida* antibodies in lung lavage. Positive and negative control TB lavages were included in each test.

3.9.1. Agar gel diffusion test

This test is prepared after the method described by Beard (1970).

3.9.1.1. Preparation of agar gel

A weight of 1 gram of agarose and 0.8 gram of sodium chloride (NaCl) were added to 100 ml of deionized distilled water (D.D.W) in flask. The mixture was then boiled in microwave for 2 min until it become clear. A volume of 0.5 ml phenol was then added as preservative. The gel was distributed in 20 ml amounts in plates and after solidification at room

temperature on a leveling table, the plates were kept in the refrigerator at 4ºC until used.

3.9.1.2. Test procedure

A rosette of six peripheral wells and central well were cut in the agar with template and agar plugs were removed with a Pasteur pipette. The central well was filled with 50µl whole cell lysate using micropipette (Antigen was mixed with 1% sodium Dodecyle sulphate (SDS)), while the peripheral wells were filled with 50µl the the tracheobronchial lavage. The plates were then covered and incubated at room temperature in humid chamber and examined daily for the development of precipitation lines. Positive results plates were then washed with NS, dried at 56 °C for 3 days, stained with Commassie blue for better visualization and pictured.

3.9.2. Indirect haemagglutination test

3.9.2.1. Tanning erythrocytes

Sheep erythrocytes (SRBCs) in Alsever's solution were washed three times in saline at 300×g for 10 min. SRBCs suspension was adjusted to 4% v/v in phosphate-saline buffer pH 7.5. Then 2.5 mg of tannic acid was added to 50 ml of phosphate buffer saline and mixed with 50 ml of 4% SRBC suspention. The mixture was incubated at 37˚C for 15 minutes. The cells were centrifuged very gently at 100×g for 20 min. Cells were divided into two aliquots, each was washed with phosphate buffered saline at 100×g for 20 min. One volume of the cells was used for coating of RBCs with antigen and the other as control cells. The cells were re-suspended in 50 ml PBS and 50 ml of the *P.multocida* whole cell antigen were added (2 mg /ml initial concentration) and incubated at 37˚C for 30 minutes and then washed in PBS by centrifugation and re-suspended in 100 ml of boarate-succinate buffer. The second aliquot of cells was re-suspended in 100 ml of boarate-

Table (2.1): Vaccination regiment of the various groups

Group	Adjuvant	Vaccine adjuvant ratio	Initial dose Day one	Booster dose day 15
G1a	Olive oil	1:1[a]	30µl	20µl
G1b		2:1[b]	30µl	20µl
G2a	Sesame oil	1:1[a]	30µl	20µl
G2b		2:1[b]	30µl	20µl
G3a	Vitamin D3	1:1[a]	30µl	20µl
G3b		2:1[b]	30µl	20µl
G4	–	10^9cfu/ml	30µl	20µl
G5	–	–	30µl	20µl

Key:
a = 5×10^8 cfu
b = 3.3×10^8 cfu

succinate buffer. The uncoated cells were used as control cells in the assay. Formalin was added to both cell suspensions to give final concentration of 0.2% formalin as a preservative. The formalin was added drop by drop during 20-30 min. Cells were left to settle for 24 hrs at 4°C and the supernatant were poured off and re-suspended in boarate-succinate buffer. The cells were allowed to settle for 24 hrs and washed again in boarate-succinate buffer. Cell suspensions were adjusted to 1% v/v.

3.9.2.2. Indirect haemagglutination test procedure

The test was performed according to Hudson and Hay (1980): Starting dilution was prepared in test tubes prior to the test using 10µl of tracheobronchial lavage diluted in 1,990µl of PBS to give final dilution of 1/200. The test was performed in v-shaped microtiter plates. Each well in microtiter plates contains 50 µl of PBS, each tracheobronchial lavage sample was two fold diluted across each row. To all wells were added with 50µl of antigen-adsorbed/sensitized red blood cells (RBCs). Positive, negative control lavages were included for each test run. Haemagglutination was detected by observing the presence or absence of tear-shaped streaming of RBCs.

3.10. Statistical analysis

Data was entered in and analyzed using the SAS program JMP version 7.0. Numerical data were presented as means and standard deviation. The significance of the mean difference between two independent groups was determined using Students' sample t test or one-way ANOVA (or ANOVA with Multiple comparisons) when comparing more than two groups. Dunnett's test was used when comparing several groups to a control group, with a p value of ≤ 0.05 considered statistically significant.

CHAPTER THREE

RESULTS

4.1. Indirec haemagglutination tests

4.1.1. Group (1)

Tracheobronchial (TB) lavages from immunized rats were tested for the presence of mucosal specific *P.multocida* antibodies by indirect heamagglutination IHA test and their titers are shown in table (3.1, 3.2). The titers of Abs for sub-group 1(a), that receive olive oil vaccine mixture 1:1, ranged between 0.256×10^5 to 2.048×10^5, while for sub-group 1(b), in which the HS vaccine and olive oil were mixed in 2:1 ratio the titers were between 0.512×10^5 to 2.048×10^5 as shown in fig (3.1) and fig (3.2). These titers were statistically (P= 0.0082) significant when compared with group 4 as depicted in fig (3.3).

4.1.2. Group (2)

The indirect heamagglutination test for the presence of specific *P. multocida* antibodies in TB lavage in sub-group 2(a) of rats immunized intranasally with HS vaccine in sesame oil in 1:1 ratio, revealed mean IHA antibody titer equal to 20.48×10^5, whereas sub-group 2(b), immunized with HS vaccine in sesame oil in 2:1 ratio, their mean IHA titer was 5.12×10^5 as shown in table (3.1, 3.2) and their fig (3.4) and (3.5). These Ab titers of sub-group 2(a) were statistically (P=0.0227) significant when compared with group (4) as depicted in fig (3.6).

4.1.3. Group (3)

Indirect haemagglutination Ab titers for TB lavage of rats in group (3) immunized with HS vaccine in vitamin D3 in 1:1 and 2:1 ratio were

shown in table (3.1). In sub-group 3 (a), the titer of Abs ranged between 2.56×10^5 and 20.48×10^5, whereas in sub-group 3(b) the Ab titers ranged between 2.56×10^5-10.24×10^5 as shown in table (3.1, 3.2), and in fig (3.7, 3.8 and 3.9). These Abs titers of sub-group 3(a) are statistically (P=0.0272) significant when compared with group (4) as depicted in fig (3.9).

4.1.4. Group (4)

Tracheobronchial lavages from this group were tested for the presence of mucosal antibodies using indirect haemagglutination test. In rats that were immunized with HS vaccine alone, the titer of Abs were ranged between 2.56×10^5 to 5.12×10^5 as depicted in table (3.1, 3.2) and fig (3.10). These Abs titer values were lower than in the other groups immunized with adjuvants as shown in fig (3.9) and (3.12) and table (3.3) and (3.4).

4.1.5. Group (5)

As demonstrated in table (5), the results of indirect haemagglutination test for lungs lavage of rats immunized with PBS as control group, were found to be negative for Abs as shown in fig (3.1), (3.2), (3.4), (3.5), (3.7), (3.8), (3.10) and (3.11).

4.1.6. The effect of vaccine adjuvant ratio on Ab titer

Vaccine adjuvant ratio 1:1 gave high Ab titer when sesame oil and vitamin D3 were used as mucosal adjuvants, whereas vaccine adjuvant ratio 2:1 gave high Ab titer when only olive oil was used as mucosal adjuvant as shown in table (3.2, 3.6) and fig (3.13). The vaccine without an adjuvant gave low Ab titer as shown in table (3.2), (3.4), (3.5) and (3.6).

4.2. Agar gel diffusion test

Tracheobronchial lavages from immunized rats were tested for the presence of mucosal specific *P.multocida* antibodies by agar gel diffusion test. Tracheobronchial lavage of all groups were found positive for the

presence of antibodies specific for *P.multocida* whole cell lysate Ag by forming sharp precipitation lines as shown in figure (3.15).

Table (3.1): Reciprocal of IHA titer for tracheobronchial lavage of the individual rat in various groups $\times 10^4$

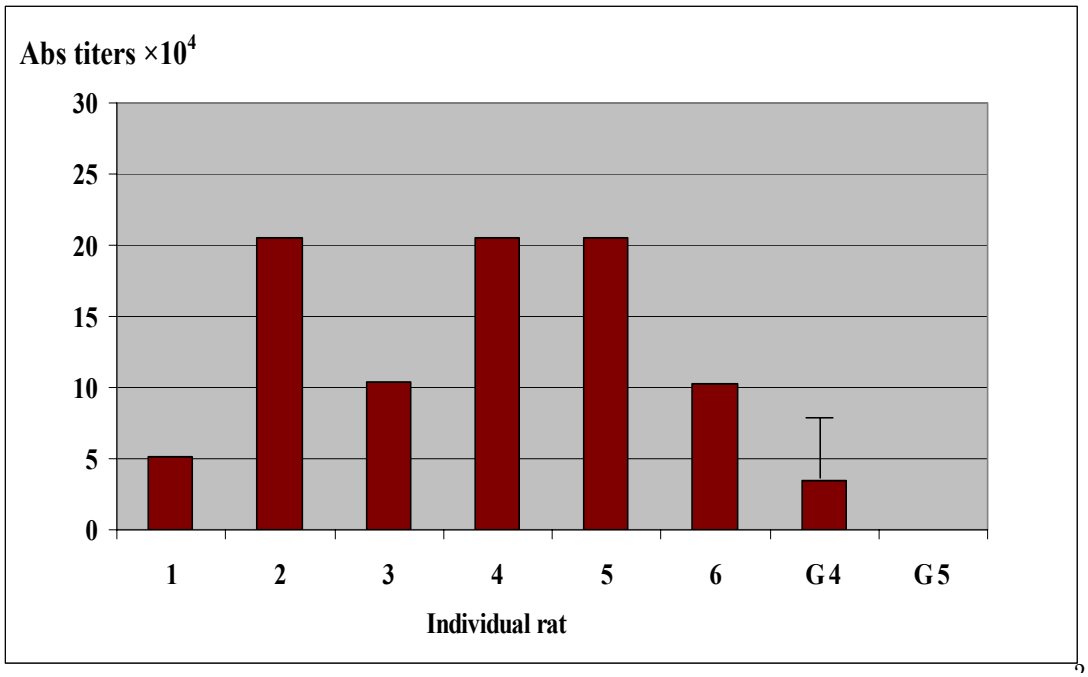
Group	Titer of Abs						Mean	Standard deviation
1 (a)	10.24	5.12	2.56	20.48	10.24	10.24	9.81	6.15
1 (b)	5.12	20.48	10.24	20.48	20.48	10.24	14.51	6.81
2 (a)	2.56	10.24	20.48	5.12	20.48	20.48	13.23	8.32
2 (b)	5.12	2.56	2.56	1.28	1.28	1.28	2.35	1.50
3 (a)	20.48	10.24	5.12	20.48	10.24	2.56	11.52	7.55
3 (b)	5.12	5.12	5.12	5.12	10.24	2.56	5.55	2.52
4	2.56	5.12	2.56	5.12	2.56	2.56	3.41	1.32
5	–	–	–	–	–	–	–	–

Table (3.2): Effect of vaccine adjuvant ratio on Abs titer $\times 10^4$

Adjuvant	Group (a)[1]	Group (b)[2]
Olive oil	9.81± 6.15	14.51± 6.81
Sesame oil	13.23± 8.32	2.35± 1.50
Vitamin D_3	11.52± 7.55	5.55± 2.52
Plain vaccine	3.14±1.32	_

Titer of Abs: mean ± standard deviation
1– Vaccine adjuvant ratio was 1:1
2– Vaccine adjuvant ratio was 2:1

Fig (3.1): Reciprocal of IHA titers of tracheobronchial lavage obtained from individual rat in group 1(a)



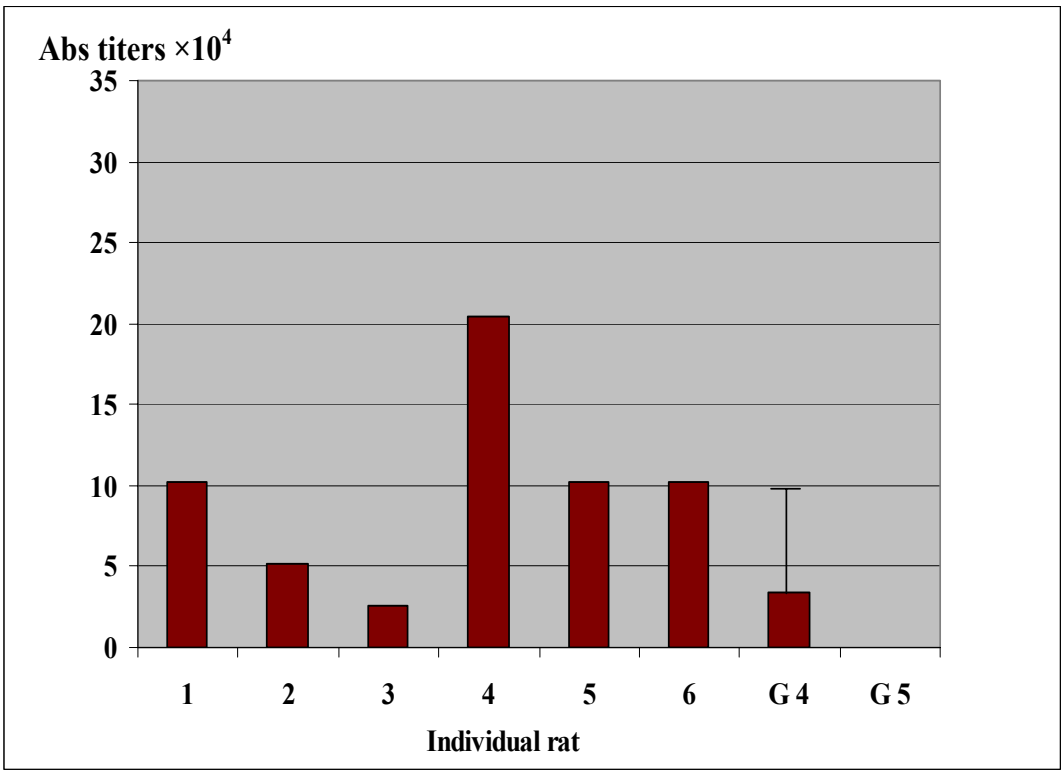
–Six rats were immunized with HS vaccine in olive oil in the ratio 1:1, induced is not statistically significant Ab titer (P= 0.2235).

–P <0.05 is significant.

–Average ± SD of IHA titer of six rats in G4 that received plain HS vaccine (without an adjuvant–adjuvant control).

–Rats in G5 were non-immunized.

Fig (3.2): Reciprocal of IHA titers of tracheobronchial lavage obtained from individual rat in group 1(b)



–Six rats were immunized with HS vaccine in olive oil in the ratio 2:1, induced a significant Ab titer (P= 0.0082).

–P <0.05 is significant.

–Average ± SD of IHA titer of six rats in G4 that received plain HS vaccine (without an adjuvant–adjuvant control).

–Rats in G5 were non□immunized.

Fig (3.3): Comparison of mean IHA titer of G1 (vaccine with olive oil) and G4 (vaccine only)

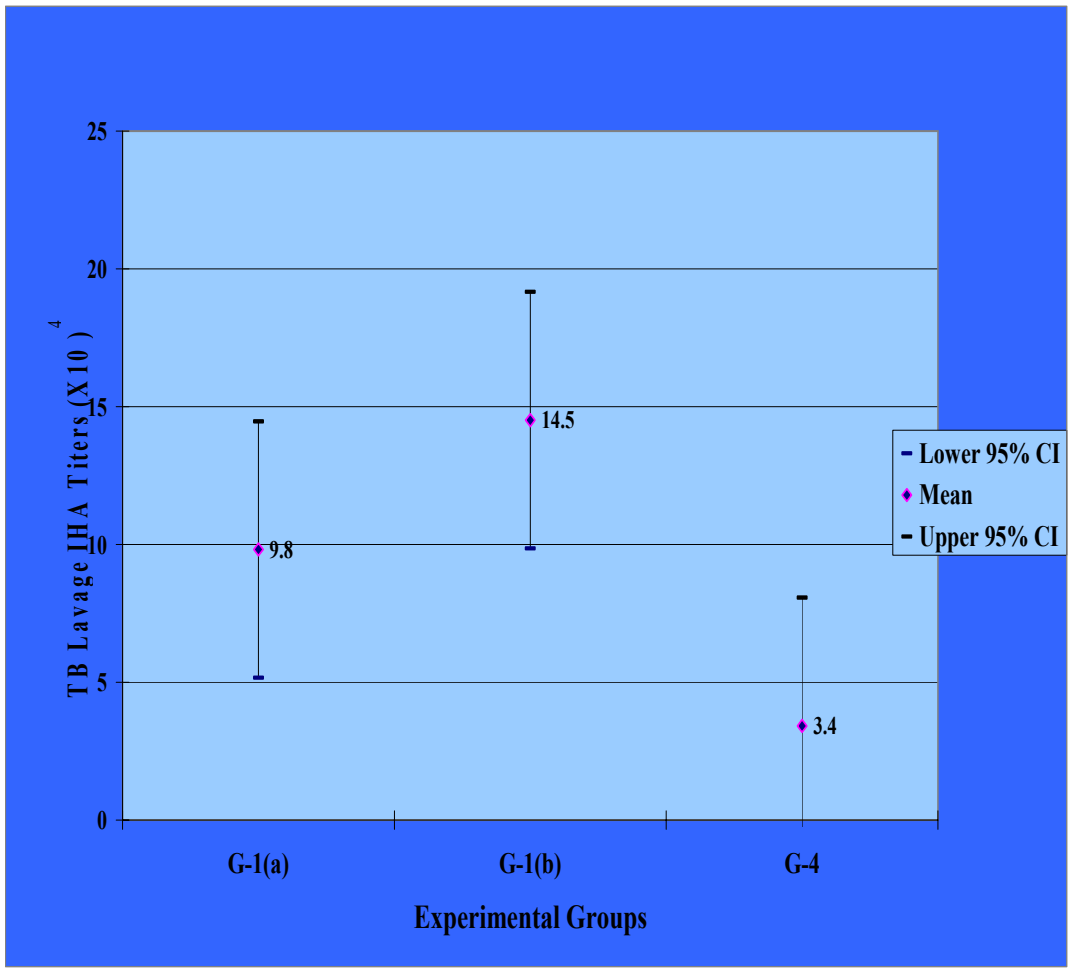
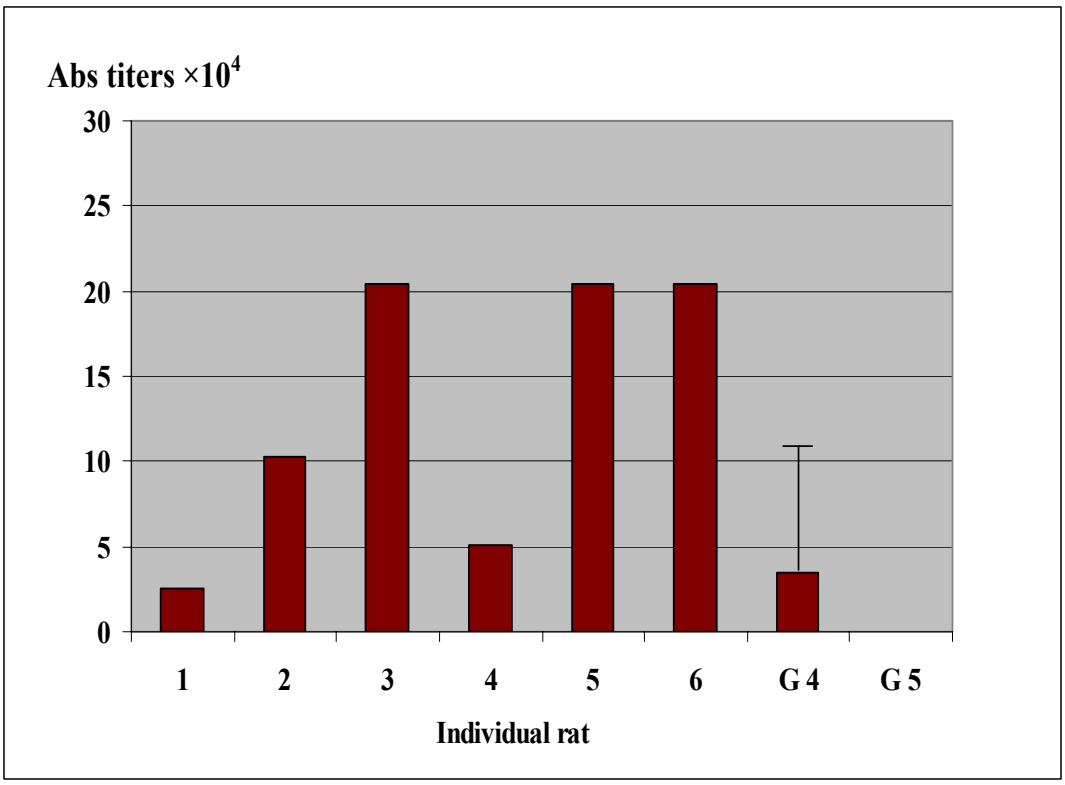


Fig (3.4): Reciprocal of IHA titers of tracheobronchial lavage obtained from individual rat in group 2(a)



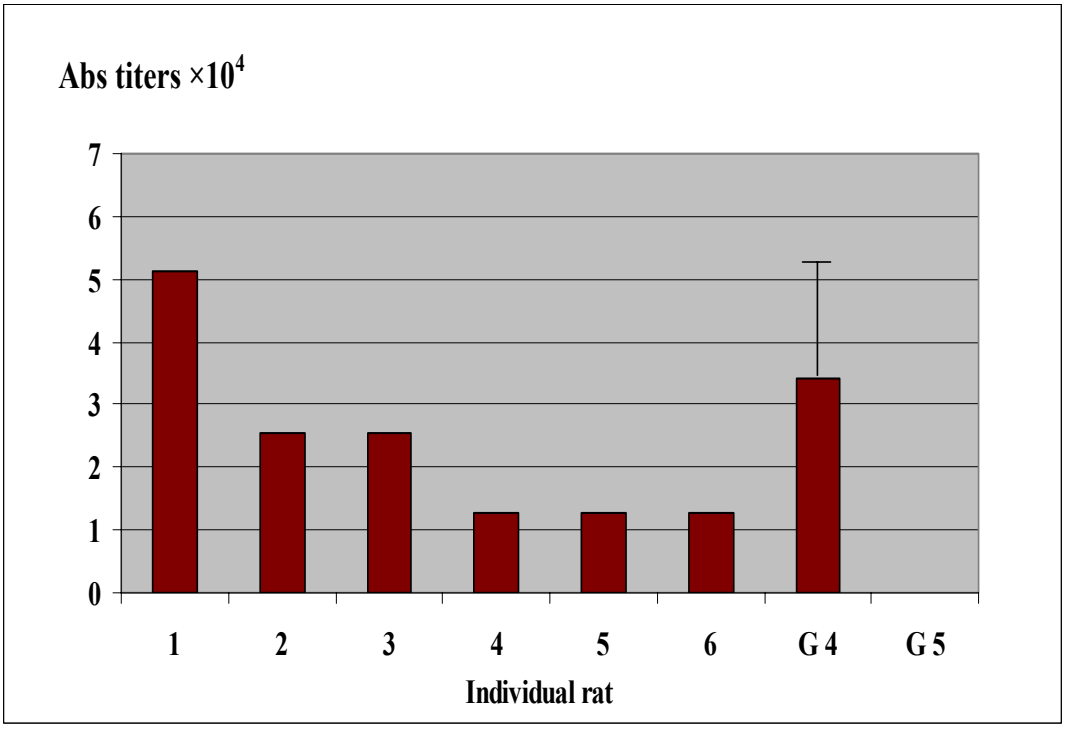
–Six rats were immunized with HS vaccine in sesame oil in the ratio 1:1, induced a significant Ab titer (P= 0.0227).

–P <0.05 is significant.

–Average ± SD of IHA titer of six rats in G4 that received plain HS vaccine (without an adjuvant–adjuvant control).

–Rats in G5 were non□immunized.

Fig (3.5): Reciprocal of IHA titers of tracheobronchial lavage obtained from individual rat in group 2(b)



–Six rats were immunized with HS vaccine in sesame oil in the ratio 2:1, Ab titer induced is not statistically significant (P= 0.9991).

–P <0.05 is significant.

–Average ± SD of IHA titer of six rats in G4 that received plain HS vaccine (without an adjuvant–adjuvant control).

–Rats in G5 were non□immunized.

Fig (3.6): Comparison of mean IHA titer of G2 (vaccine with sesame oil) and G4 (vaccine only)

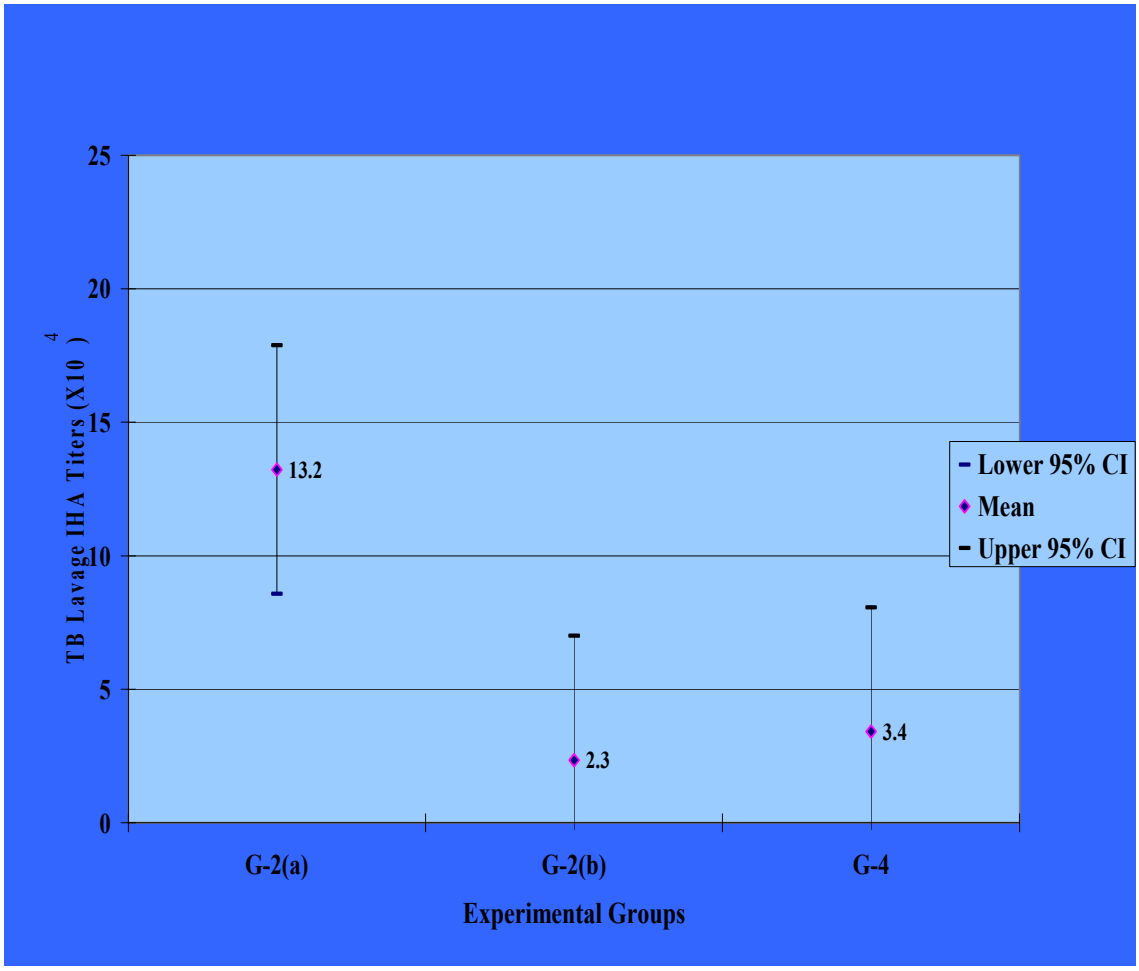
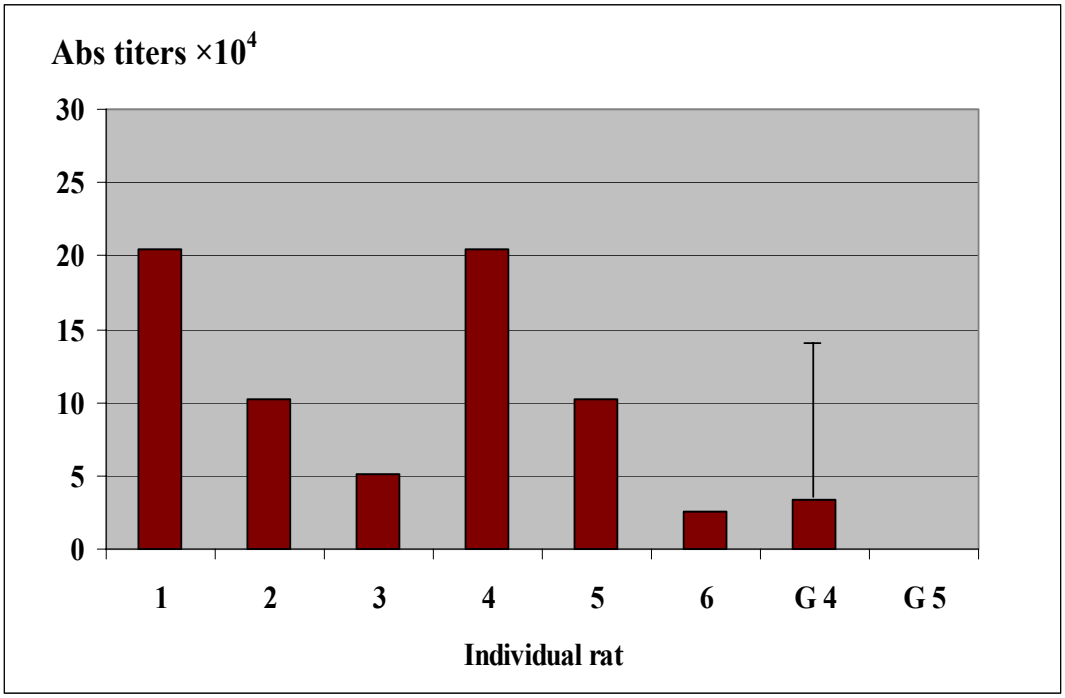


Fig (3.7): Reciprocal of IHA titers of tracheobronchial lavage obtained from individual rat in group 3(a)



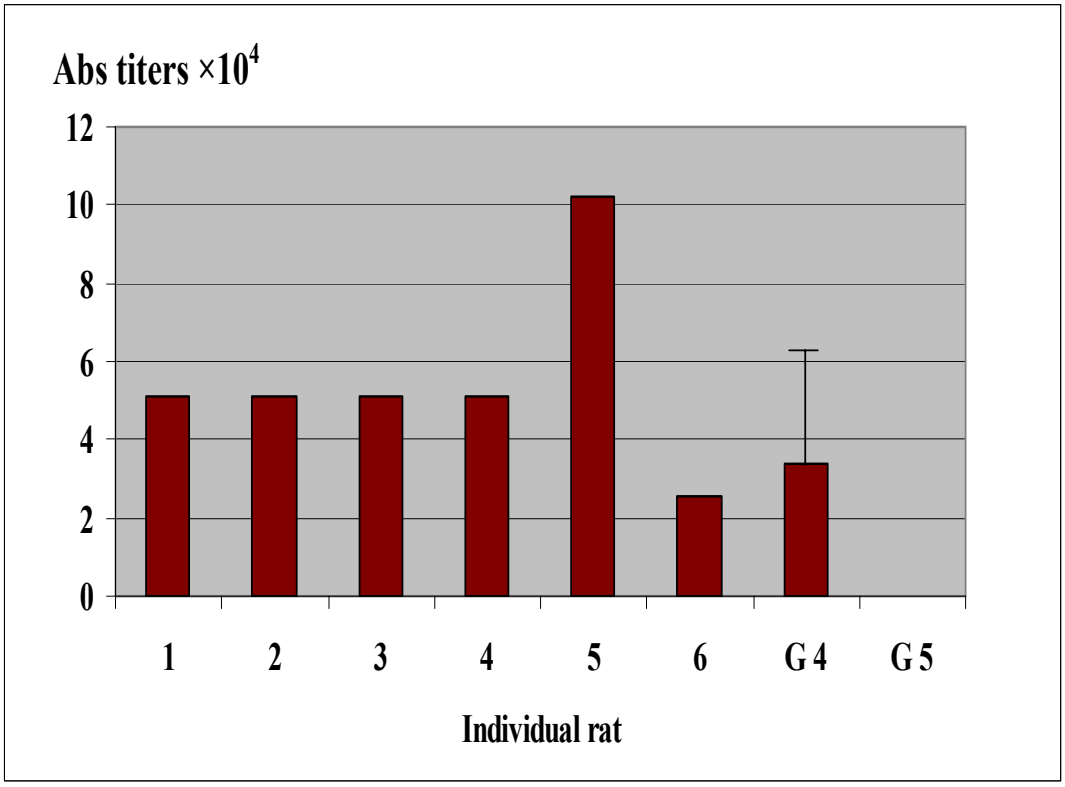
–Six rats were immunized with HS vaccine in vitamin D_3 in the ratio 1:1, induced a significant Ab titer (P= 0.0272).

–P <0.05 is significant.

–Average ± SD of IHA titer of six rats in G4 that received plain HS vaccine (without an adjuvant–adjuvant control).

–Rats in G5 were non□immunized.

Fig (3.8): Reciprocal of IHA titers of tracheobronchial lavage obtained from individual rat in group 3(b)



–Six rats were immunized with HS vaccine in vitamin D_3 in the ratio 2:1, Ab titer induced is not statistically significant (P= 0.9661).

–P <0.05 is significant.

–Average ± SD of IHA titer of six rats in G4 that received plain HS vaccine (without an adjuvant–adjuvant control).

–Rats in G5 were non□immunized.

Fig (3.9): Comparison of mean IHA titers of G3 (vaccine with vitamin D3) and G4 (vaccine only)

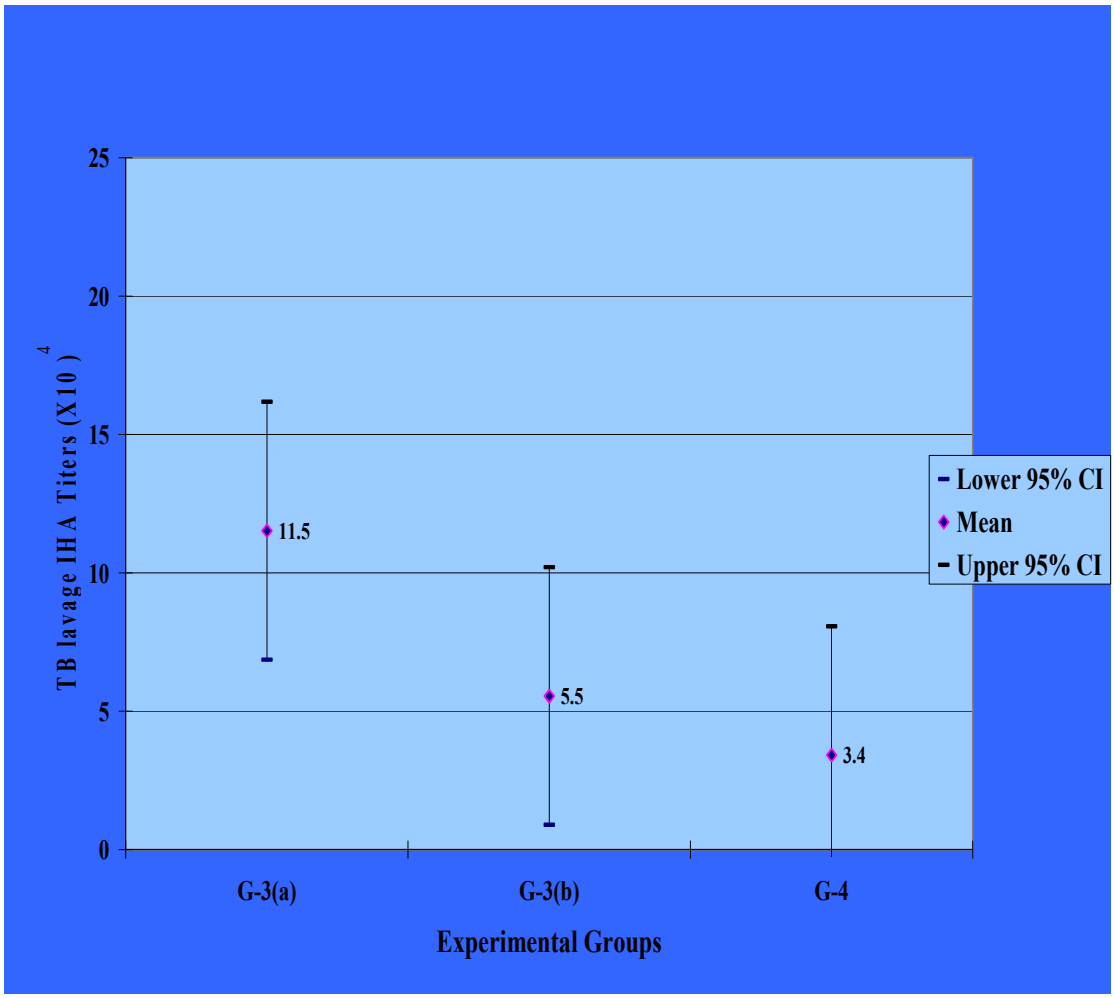
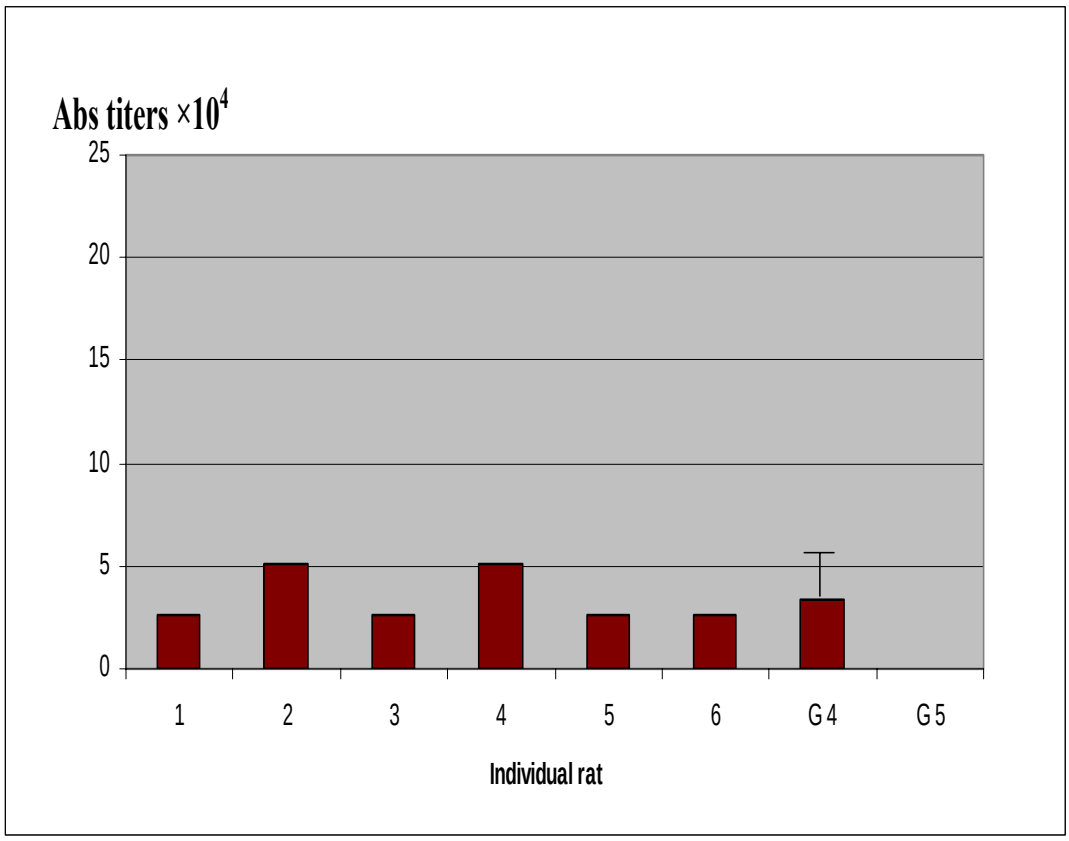


Fig (3.10): Reciprocal of IHA titers of tracheobronchial lavage obtained from individual rat in group 4

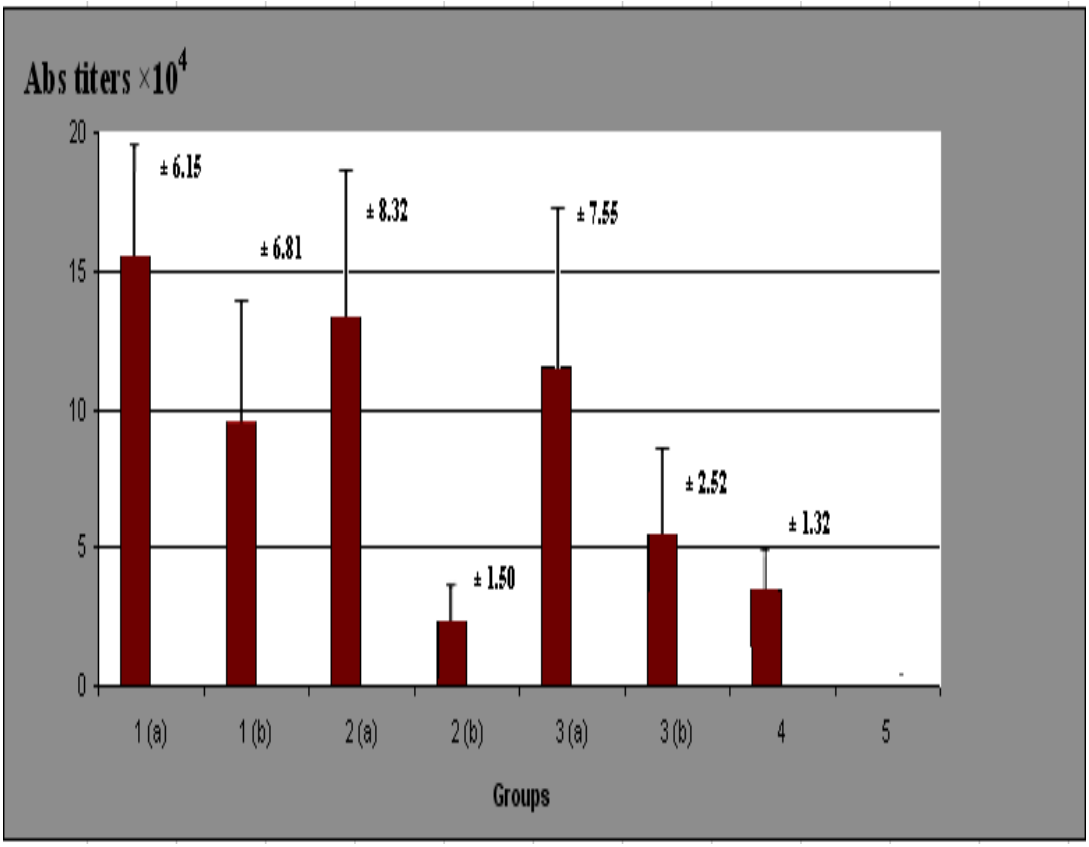


–Six rats were immunized with HS vaccine alone.

–Average ± SD of IHA titer of six rats in G4 that received plain HS vaccine (without an adjuvant–adjuvant control).

–Rats in G5 were non-immunized.

Fig (3.11): Reciprocal of IHA titers for tracheobronchial lavage in various groups

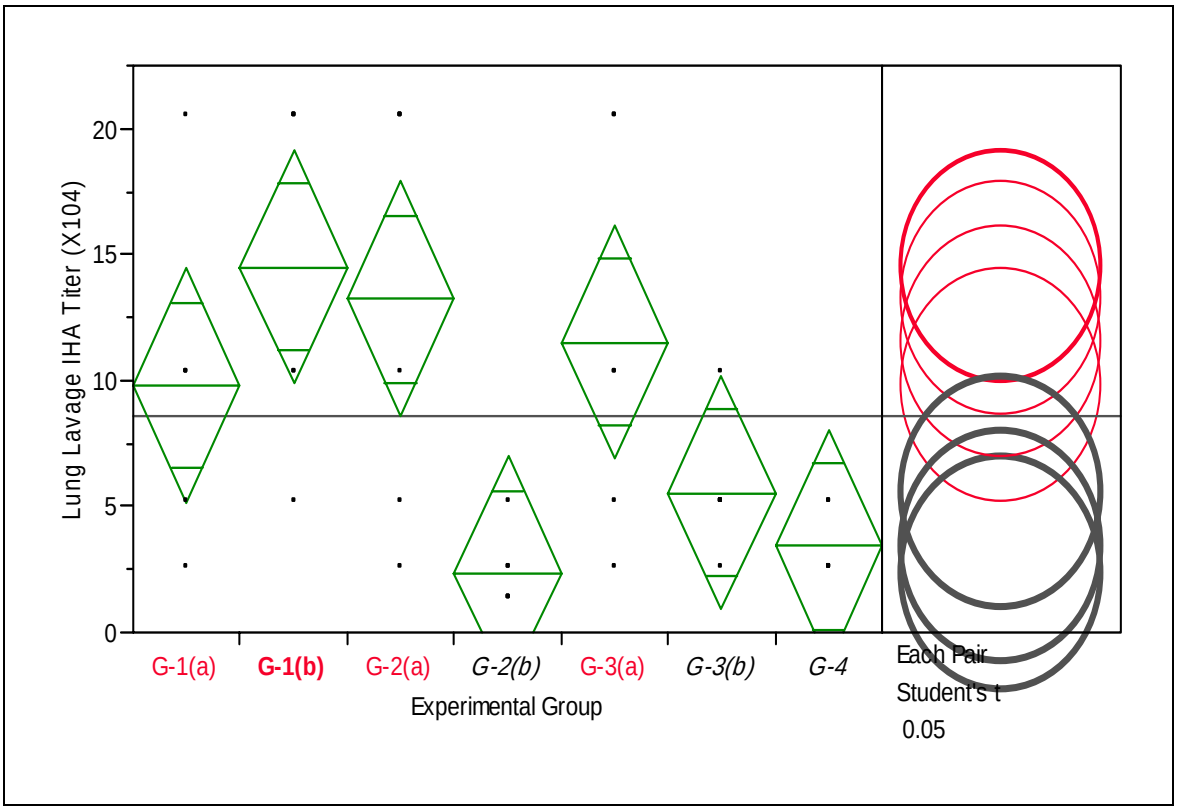


- Bar ±SD represent mean ± SD.
- Ab titers were significant in group: G1a, G1b, G2a and G3b.

4.3. Visual Comparison of Group Means

Each multiple comparison test begins with a comparison circles plot, which is a visual representation of group mean and 95% Confidence Interval comparisons. The plot follows with a reveal table of the comparisons. Overlap marks show for each diamond and are computed as (group mean ± CI). Overlap marks in one diamond that are closer to the mean of another diamond than that diamond's overlap marks indicate that those two groups are not different at the 95% confidence level. You can compare each pair of group means visually by examining how the comparison circles intersect. The outside angle of intersection tells you whether group means are significantly different. Circles for means that are significantly different either do not intersect or intersect slightly so that the outside angle of intersection is less than 90 degrees. If the circles intersect by an angle of more than 90 degrees or if they are nested, the means are not significantly different. If the intersection angle is close to 90 degrees, you can verify whether the means are significantly different by clicking on the comparison circle to highlight it. The highlighted circle appears with a thick solid line. Red circles representing means that are not significantly different from the highlighted circle show with thin lines. Circles representing means that are significantly different show with a thick gray pattern.

Fig (3.12): Visual comparison of mean and 95% CI of IHA titer ($\times 10^4$) among all rat groups

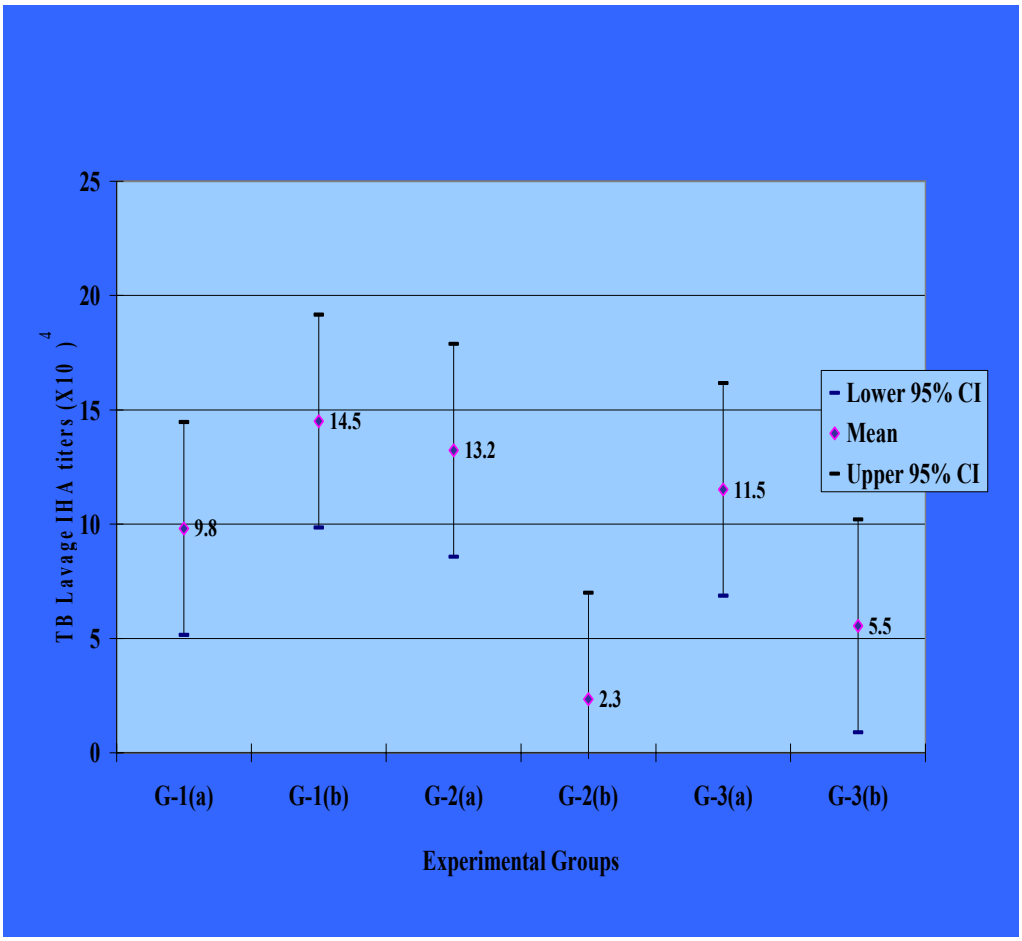


G-1(a): Vaccine + Olive Oil (1:1), G-1(b): Vaccine + Olive Oil (2:1), G-2(a): Vaccine + Sesame Oil (1:1), G-2(b): Vaccine + Sesame Oil (2:1), G-3(a): Vaccine + Vitamin D_3 (1:1), G-3(b): Vaccine + Vitamin D_3 (2:1).

(F ratio=4.5, p value=0.001, statistically significant)

Fig (3.13): Effect of vaccine-adjuvant ratio on IHA titres of *P. multocida* in immunized rats.

Reciprocal of IHA titers for tracheobronchial lavage



Comparison of mean and 95% CI of tracheobronchial lavage IHA titers among the experimental groups.

(Statistically significant, p=0.001)

Table (3.3): Means and 95% CI for One-way ANOVA test

Experimental Group	Mean	95% Confidence Interval	
		Lower Bound	Upper Bound
G-1(a)	9.81	5.48	14.15
G-1(b)	14.51	10.17	18.84
G-2(a)	13.23	8.89	17.56
G-2(b)	2.35	-1.99	6.68
G-3(a)	11.52	7.19	15.85
G-3(b)	5.55	1.21	9.88
G-4	3.41	-0.92	7.75

Table (3.4): Comparison of each group with the control group using Dunnett's method

Control Group	Experimental Group	p-Value
G-4	G-1(b)	0.0082*
	G-2(a)	0.0227*
	G-3(a)	0.0272*
	G-1(a)	0.2235
	G-3(b)	0.9661
	G-2(b)	0.9991

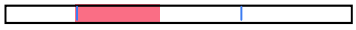
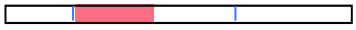
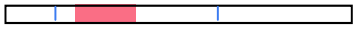
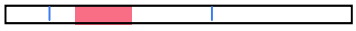
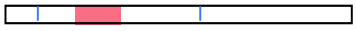
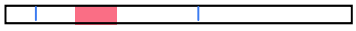
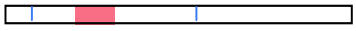
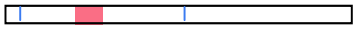
*statistically significant at p value ≤ 0.05

Table (3.5): Comparison of each pair using Students' Sample t test: Connected letters report

Levels not connected by same letter are significantly different.

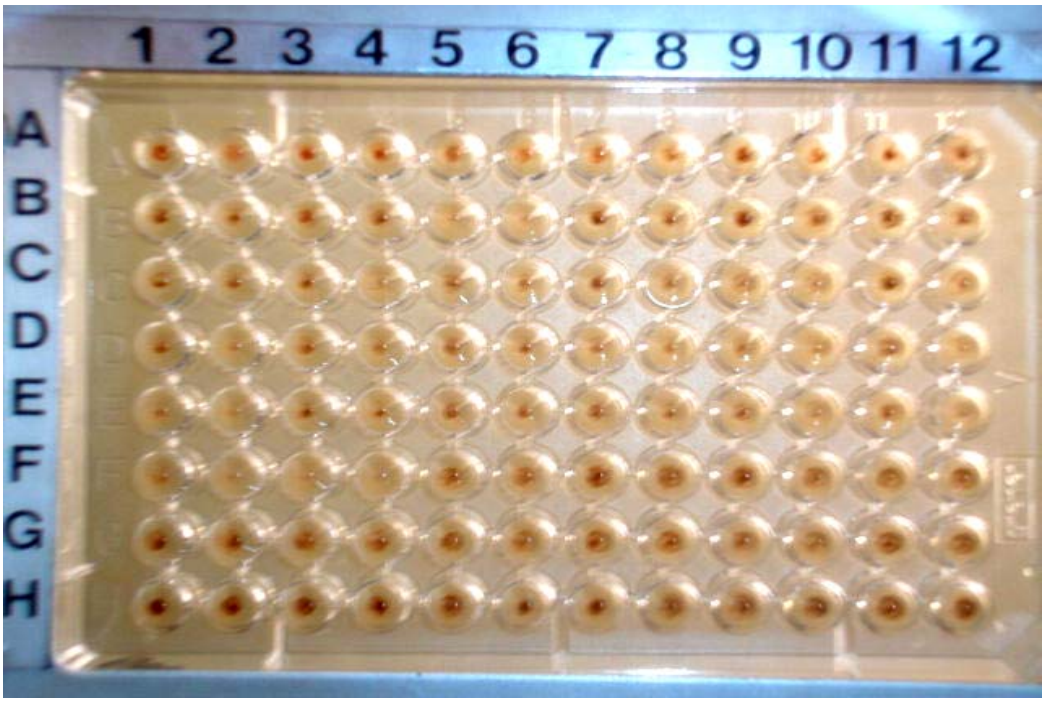
Group					Mean
G-1(b)	A				14.51
G-2(a)	A				13.23
G-3(a)	A	B			11.52
G-1(a)	A	B	C		9.81
G-3(b)		B	C	D	5.55
G-4			C	D	3.41
G-2(b)				D	2.35

Table (3.6): Ordered differences report between the various immunized groups

Comparison		Difference	P value	Difference
G-1(b)	G-2(b)	12.16	0.0006*	
G-1(b)	G-4	11.09	0.0016*	
G-2(a)	G-2(b)	10.88	0.0019*	
G-2(a)	G-4	9.81	0.0046*	
G-3(a)	G-2(b)	9.17	0.0077*	
G-1(b)	G-3(b)	8.96	0.0090*	
G-3(a)	G-4	8.11	0.0172*	
G-2(a)	G-3(b)	7.68	0.0235*	
G-1(a)	G-2(b)	7.47	0.0273*	
G-1(a)	G-4	6.40	0.0563	
G-3(a)	G-3(b)	5.97333	0.0739	
G-1(b)	G-1(a)	4.69333	0.1566	
G-1(a)	G-3(b)	4.26667	0.1967	
G-2(a)	G-1(a)	3.41333	0.2996	
G-3(b)	G-2(b)	3.2	0.3304	
G-1(b)	G-3(a)	2.98667	0.3632	
G-3(b)	G-4	2.13333	0.5148	
G-2(a)	G-3(a)	1.70667	0.6019	
G-3(a)	G-1(a)	1.70667	0.6019	
G-1(b)	G-2(a)	1.28	0.6954	
G-4	G-2(b)	1.06667	0.7441	

*statistically significant at p value ≤ 0.05

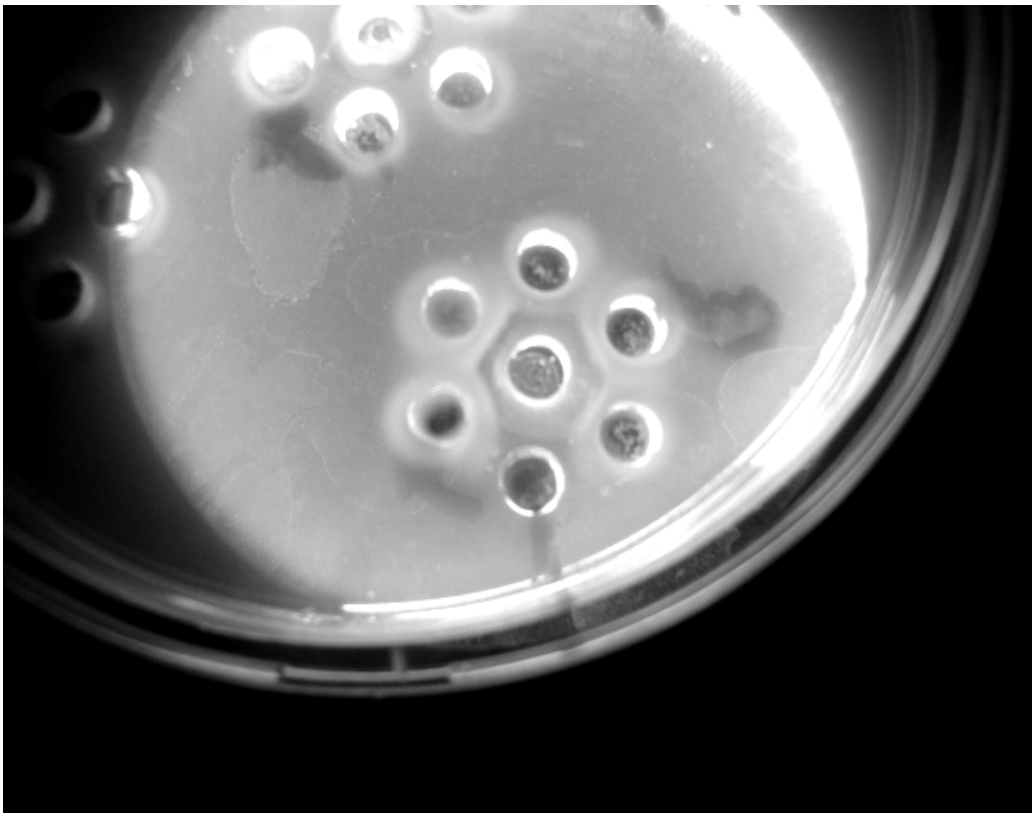
Fig (3.14): Indirect haemagglutination test in microtiter plate.



–The end points of Ab titre is the highest dilution of tracheobronchial lavage that gave visible positive agglutination of RBCs.

–In haemagglutination negative wells RBCs form a button at the bottom of the well.

Fig (3.15): Agar gel precipitation test.



–Prepheral wells were filled with tracheobronchial lavage of individual rat.

–Central well contained *P. multocida* whole cell lysate.

–Precence of mucosal specific *P. multocida* antibodies is indicated by the formation of strong precipitation lines (band).

–Continuity of the line indicates mucosal Ab recognize a common Ag of *P. multocida* whole cell lysate.

CHAPTER FOUR
DISCUSSION

The mucosal surface of the respiratory tract represents one of the principle portals of entery for most animal pathogen. Most of mucosal surfaces are replete with organized follicles and scattered antigen reactive or sensitized lymphocytes (Macpherson, McCoy, Johansen and Boandzaeg, 2007). Therefore, immune response to mucosal pathogens generates effectors humoral and cell-mediated immune responses, which may lead to protection at mucosal surface. However, the control of haemorrhagic septicaemia remains a problem because the current vaccines are not sufficiently efficacious and require repeated administration and most of them are administered parenterally and may not effectively induce mucosal immune response in the respiratory tract (Smith, Babiuk and Stockdale, 1981; Jaffari, Gill, Bhatti and Raza, 2006 and Cox *et al*., 2006). For instance, the bactrins made from *P.multocida* have been available for many decades but with little effect in preventing the disease. Nonetheless, investigation into the immunogenic potential of *P.multocida* over the last several years have led to the development of a variety of immunoprophylactic agents but none of these has yet replaced the old bactrin vaccines.

In this study, the primary reason for using intranasal route of immunization is that *P.multocida* infection affects or initiates the infectious process at the mucosal surface of the upper respiratory tract and mucosal application of *P.multocida* broth bactrin is required to induce a local antibody response. For most of respiratory tract infections, the induction of local immunity is crucial for optimal protection (Cox *et al*., 2006). The

induction of SIgA represents the main effectors mechanism of the local adaptive immune response and thus represents the primary goal for mucosal vaccine (Gerdts *et al.*, 2006). This implicates that vaccination strategies should induce effectors mechanisms which help to prevent *P.multocida* establishment and their subsequent invasion in the first place. It is believed that one way of achieving this goal is vaccination through mucosal surface itself in order to generate an effective local antibody response. This is inconsistent with the findings of Zamri-saad, Effendy, Israf and Azmi. (1999) who reported that intranasal immunization not only induces a local immune response, but also prime systemic responses and mimics the oronasal route of infection in natural disease.

The present study was designed to evaluate the immunogenicity of *P.multocida* in rats using intranasal route of immunization and the effect of mucosal adjuvants as well as vaccine-adjuvant ratio. High antibody activity was detected in TB lavage obtained from intranasally immunized rats and found to be specific to *P.multocida* by indirect haemagglutination assay (IHA) and gel diffusion precipitation test (GDPT) (fig 3.14 and 3.15).

In GDPT sharp precipitation lines were obtained using TB lavage from *P.multocida* immunized rats of G1b, G2a and G3a, that received the broth bactrin-adjuvant mixture as depicted in fig (3.15). These finding are in agreement with the findings of Suckow, Boweosock, Nielsen, and Grigdesby (1996) who demonstrated antibody activity in TB lavage of rabbit immunized with co-administration of potassium thiocyanate extract of *P.multocida* and cholera toxin as an adjuvant, than with the extract alone. These authors also reported protective effect of these locally produced Ab to challenge. Moreover, IHA assay revealed high Ab titres in TB lavages of rats that received *P. multocida* broth bactrin adjuvant mixture fig (3.3), (3.6)

and (3.9). These findings substantiate the results of Jarvinin, Hogenesch, Suckow, and Bowersock, (1998) who induced a protective local antibody response in rabbits. Furthermore, Smith *et al*., (1981) assumed that the route of natural infection by *P. multocida* is via the respiratory tract and he suggested that local defence mechanisms were important in preventing the establishment of infection. In this study, rats of G1b that received *P. multocida* broth bactrin in olive oil in ratio 2:1 gave high Ab activity in their TB lavage as determined by IHA assay in comparison to G4 that received the plain broth bactrin vaccine (P=0.0082) as shown in table (3.1), (3.3), (3.4), (3.5) and (3.6) and fig (3.2), (3.3) and (3.12).

Similar high Ab activity was demonstrated in TB lavage of rats of G2a and G3a that received *P. multocida* broth bactrin in sesame oil and vitamin D3 in the ratio 1:1 respectively, in comparison to rats of G4 ($P \leq 0.005$) as shown in table (3.1), (3.2), (3.3) and (3.4) and fig (3.4), (3.6), (3.7), (3.9) and (3.12).This local high Ab activity in TB lavage agrees with the finding of previous studies that showed mucosal but not subcutaneous immunization had resulted in high production of Ab response of SIgA isotype (Butler, Swanson, Richerson, Ratajczat, Richards and Suelzer, 1982 and Suckow, Boweosock, Nielsen and Grigdesby, 1996). Furthermore, intranasal immunization caused an increased IgA Abs level in nasally associated lymphoid tissue, in the upper respiratory tract, as well as the bronchial-associated lymphoid tissue (Lu, Pakes, Massey and Slefann, 1987). The presence of IgA Abs has been shown to be important in blocking bacterial adhesion and antigen uptake across the mucosal membrane (Suckow *et al.*, 1996 and Macpherson *et al*., 2007). Thus, if *P.multocida* could be eliminated at the site of infection by locally produced antibodies, then further systemic damage and possible death can be prevented. Therefore, induction of a

distinct IgA isotype that dominates mucosal antibodies constituted a strong evidence for local immune system structurally and functionally distinct from systemic compartment (Macpherson *et al.*, 2007). Studies that compared humoral immunity at mucosal surface with serum immune response had repeatedly shown a separation between secretory and systemic immune responses (Suckow *et al*., 1996; and Cox *et al*., 2006). Mucosal surfaces represent a critical component of mammalian immunologic repertoire. The major Ab isotype in external secretion is SIgA and approximately 40 mg of IgA is produced by kg body weight and IgA synthesis is about twice the a mount of IgG produced daily in man (Conley and Delacroix, 1987). This could explain the high IHA titre obtained in TB lavage of intranasally immunized rats as depicted in table (3.1), (3.2), (3.3) and (3.4) and fig (3.1), (3.2), (3.4), (3.5), (3.7) and (3.8).

Intranasal immunization has been shown to induce expression of L-selectin as well as $\alpha\text{-}_4\beta_7$ integrin on sensitized lymphocytes to favour their mucosal homing (Cox *et al*., 2006; Gerdts *et al*., 2006, and Macpherson *et al*., 2007). In this study, the booster dose of *P. multocida* broth bactrin was found necessary for prolonged and strong response as the vaccine is dead vaccine and the immune response that follows a single inoculation of a dead antigen usually falls to a negligible level within few weeks, but it can be rapidly revived by secondary or booster dose as has been suggested in this study. Furthermore, since the effectiveness of *P. multocida* broth bactrin vaccine is expected to be much lower than live vaccine, we incorporated mucosal adjuvant as a means of a delivery system that was needed to enhance the uptake of the vaccine antigen and increase the induction of local immune response fig (3.3), (3.6), (3.9), (3.10), (3.11) and (3.12). From this study, it seems that intranasal immunization induces high Ab titre in the

respiratory tract of rats as it was reported by Jarvinen, Hogenesch, Suckow and Bowersock, 1998) and is an effective route to control *P. multocida* infection. Moreover, Smith, *et al.*, (1981) reported that intranasal immunization conferred better protection for intranasal challenge than did intramuscular immunization and showed an accelerated pulmonary clearance of *P. multocida* after intranasal immunization. However, the method of vaccine delivery might be viewed as impractical when vaccinating large number of animals, but the efficacy of mucosal vaccination suggests that it may be possible to eventually deliver such vaccines by an alternative route such as oral route to induce a mucosal immunity at the respiratory tract. This is inconsistent with the notion that the mucosal immune system is highly compartmentalized but is in perminant contact with each other providing a wide variety of possible route of immunizations (Macpherson *et al.*, 2007). For instance, earlier studies by Ringler, Peter and Keren, (1985) demonstrated the development of antibody activity to *P. multocida* capsular antigens in nasal lavage and sera following intranasal and conjunctival immunization. Furthermore, mucosal delivery of a vaccine could provide a safe, practical and effective means of protection in animals without concern for the development of infection site reaction, or discomfort from parenteral immunization (Cox *et al.*, 2006).

Although immunity to haemorrhagic septicaemia was demonstrated in appropriately immunized animals, there are still many unanswered questions regarding the mechanisms involved. Most of these studies tell little about the means by which the vaccinated hosts control growth of *P. multocida in vivo* at early stages of infection at the respiratory tract (Cox *et al.*, 2006). Divergent results regarding route and form of the vaccine antigen require further investigation in the light of our present findings and extrapolation

from present result and these of (Smith *et al.*, 1981; Suckow *et al.*, 1996 and Jarvinen *et al.*, 1998) tend to support our hypothesis that protection from respiratory infection is best achieved through intranasal or other mucosal route. Therefore, in developing vaccine against haemorrhagic septicaemia it seems that mucosal route and mucosal adjuvant are the key players for inducing a protective local immune response. Nonetheless, mucosal vaccination is proving to be one of the greatest challenges in modern vaccine development. Although mucosal vaccination is highly beneficial for achieving protective immunity, yet it remains a difficult task (Cox *et al.*, 2006 and Gerdit *et al.*, 2006). Adjuvants have been used for the last 8 decades to enhance the immune response of animals to vaccine antigen. Hence, the development of mucosal vaccine depends on efficient antigen delivery systems to present the vaccine antigen to the mucosal system. Therefore, the goal of vaccination is to stimulate a strong protective and long lasting immune response to the administered antigen. For achievement of these objectives, a potent adjuvant and novel vaccine antigen and vaccine strategy are required to make the vaccine sufficiently immunologic to initiate a potent immune response at the respiratory tract. Therefore, vaccination requires optimal mucosal adjuvants, including immunopotentiator and delivery system that offer long term protection from infectious diseases of the mucosal surfaces in man and animals.

CONCLUSION

1. Mucosal adjuvant plays a pivotal role in inducing a local immune response at mucosal surfaces when mixed with mucosal vaccines.
2. High Ab titre specific to *P. multocida* were present in TB lavage of intranasally immunized rats as was determined by IHA assay and GDPT.
3. Our results suggest that intranasal immunization and use of mucosal adjuvants are important delivery system for *P. multocida* derived immunogen to induce a local immune response and this is probably achieved through antigen targeting to the APC. The Ab titres in G1, G2 and G3 are statistically significant compared to rats in G4 that received plain *P. multocida* vaccine without an adjuvant.
4. *P. multocida* broth bactrin adjuvant ratio 1:1 was found optimal for sesame oil and vitamin D3 to induce high Ab titres in TB lavage of intranasally immunized rats, whereas vaccine- adjuvant ratio of 2:1 was found optimal for olive oil to induce high Ab titre compared to rats in G4.

RECOMMENDATIONS

1. Mucosal immunization should be adopted for protection of mucosal surfaces against colonization and invasiveness by pathogenic microorganisms.
2. Further investigations are required to study the efficacy of intranasal immunization to challenge with *P. multocida*.
3. Divergent results regarding routes of administration and form of *P. multocida* vaccine require further investigation, but extrapolation from our present results and those of others, in many other systems tend to support our hypothesis that protection is best achieved by immunization via intranasal route or an alternative mucosal route.

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